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TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US)

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/US99/19068	August 18, 1999	August 18, 1998

TITLE OF INVENTION

TREATMENT AND PREVENTION OF CANCER AND PITUITARY DISORDERS WITH LATS PROTEINS, DERIVATIVES AND FRAGMENTS, AND LATS KNOCK-OUT ANIMAL MODELS

APPLICANT(S) FOR DO/EO/US

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Applicant herewith submits to the United States Designated/ Elected Office (DO/EO/US) the following items under 35 U.S.C. 371:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.

2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.

3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).

4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.

5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. ☒ is transmitted herewith (required only if not transmitted by the international Bureau).
   b. ☐ has been transmitted by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US)

6. ☐ A translation of the International Application into English (35 U S.C. 371(c)(2)).

7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
   b ☐ have been transmitted by the International Bureaus.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☒ have not been made and will not be made.

8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 37(c)(3)).

9. ☒ An unexecuted oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).

10. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern document(s) or information included:

11. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.

12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.

13. ☐ A FIRST preliminary amendment.
    ☐ A SECOND or SUBSEQUENT preliminary amendment.

14. ☐ A substitute specification.

15. ☐ A change of power of attorney and/or address letter.

16. ☒ Other items or information:

    1. Copy of Preliminary Examination Report
    2. Copy of Preliminary Search Report
    3. Copy of WO 00/10602

INTERNATIONAL APPLICATION NO. PCT/US99/19068 09/763334	INTERNATIONAL FILING DATE August 18, 1999

17. ☒ The U.S. National Fee (35 U.S.C. 371(c)(1)) and other fees as follows:

CLAIMS				
(1)FOR	(2)NUMBER FILED	(3)NUMBER EXTRA	(4)RATE	(5)CALCULATIONS
TOTAL CLAIMS	115 - 20	95	X $ 18.00	$ 1,710.00
INDEPENDENT CLAIMS	22 - 3	19	X $ 80.00	1,520.00
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ $ 270.00	$ 270.00
BASIC NATIONAL FEE (37 CFR 1.492(a)(1)-(5)): **CHECK ONE BOX ONLY** ☒ International preliminary examination fee paid to USPTO (37 CFR 1.482) ..... $ 690				$ 690.00
☐ No international preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1.445(a)(2)) ..... $ 710				
☐ Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO $1000				
☐ International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2) to (4) ..... $ 100				
☐ Filing with EPO or JPO search report ..... $ 860				
Surcharge of $130.00 for furnishing the National fee or oath or declaration later than 30 mos. from the earliest claimed priority date (37 CFR 1.492(e)).				$ 130.00
		TOTAL OF ABOVE CALCULATIONS		= 4,320.00
Reduction by 1/2 for filing by small entity, if applicable. Affidavit must be filed also. (Note 37 CFR 1.9, 1.27, 1.28).				- $ 0.00
		SUBTOTAL		= 4,320.00
Processing fee of $130.00 for furnishing the English Translation later than 20 30 mos. from the earliest claimed priority date (37 CFR 1.492(f)).				+
		TOTAL FEES ENCLOSED		$ 4,320.00

a. ☐ A check in the amount of $__ to cover the above fees is enclosed.

b. ☒ Please charge Deposit Account No. 16-1150 in the amount of $__ to cover the above fees. A copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 16-1150. A copy of this sheet is enclosed.

18. ☐ Other instructions
n/a

19. ☒ All correspondence for this application should be mailed to
PENNIE & EDMONDS LLP
1155 AVENUE OF THE AMERICAS
NEW YORK, NEW YORK 10036-2711

20. ☒ All telephone inquiries should be made to (212) 790-2803

Adriane M. Antler	Adriane M. Antler	32,605	February 16, 2001
NAME	SIGNATURE	REGISTRATION NUMBER	DATE

By Margaret B. Boissonneault
Reg No. 40,922

TREATMENT AND PREVENTION OF CANCER AND PITUITARY DISORDERS WITH LATS PROTEINS, DERIVATIVES AND FRAGMENTS, AND *LATS* KNOCK-OUT ANIMAL MODELS

This invention was made with Government support under Grant number NIH-NCI 1 R01 CA 69408 awarded by the National Institutes of Health. The Government has certain rights in the invention.

RELATED APPLICATIONS

Priority is claimed to United States provisional application Serial Nos. 60/096,997 and 60/096,996, both filed on August 18, 1998, both of which are incorporated by reference herein in their entireties.

FIELD OF THE INVENTION

The present invention relates to the use of lats proteins, derivatives and fragments for the treatment of cancer, particularly for the treatment of cancer that is refractory to treatment by standard chemotherapy and radiation therapy protocols. The present invention also relates to the use of lats proteins, derivatives and fragments for the treatment of diseases and disorders associated with an aberrantly high or aberrantly low level of cdc2 activity. The present invention further provides complexes of lats and cdc2, and their production and uses. The present invention also provides an animal model for cancer, particularly for skin cancer, soft tissue sarcomas, and ovarian tumors, and for pituitary disorders. The animal model is preferably a mouse, in which a *lats* gene has been disrupted by homologous recombination, *e.g.*, a *lats* knock-out mouse. The present invention also provides methods of screening potential therapeutics for efficacy in the treatment and prevention of cancer and pituitary disorders using *lats* knock-out animals.

BACKGROUND OF THE INVENTION

Cancer

A neoplasm, or tumor, is a neoplastic mass resulting from abnormal uncontrolled cell growth, which may cause swelling on the body surface, and which can be benign or malignant. Benign tumors generally remain localized. Malignant tumors are collectively termed cancers. The term "malignant" generally means that the tumor can invade and destroy neighboring body structures and spread to distant sites to cause death (for review, see Robbins and Angell, 1976, *Basic Pathology*, 2d Ed., W.B. Saunders Co., Philadelphia, pp. 68-122). Treatment options, such as surgery, chemotherapy and radiation treatment, are

either ineffective or present serious side effects. Thus, there is a need for development of new drugs for the treatment of cancer.

The Cell Cycle and Tumor Suppressors

Many cancers have been linked to perturbations in the regulation of the cell cycle, resulting in deregulation of cell growth. Briefly, the cell cycle occurs in four stages: G1 (for Gap1), the resting stage prior to DNA synthesis; S (for synthesis) phase, in which DNA synthesis occurs; G2 (for Gap2), the resting stage after DNA synthesis and prior to mitosis; and M phase, mitosis, in which cell division occurs. Progression of the cell cycle is driven by a group of cyclin-dependent kinases (CDKs) (Elledge, 1996, Science 274:1664-1672; Nasmyth, 1996, Science 274:1643-1645). The kinase activities of CDKs require their positive subunits, the cyclins, and the activities of specific CDK/cyclin complexes are in turn positively and negatively regulated by phosphorylation events and CDK inhibitors (CKIs) (Hunter and Pines, 1995, Cell 80:225-236; Morgan, 1995, Nature 374:131-134). While the specific CDKs, CDK2, CDK4 and CDK6, along with Cyclins D and E, regulate the progression from G1 into S phase, cdc2, along with Cyclins A and B, regulate the cell cycle progression from G1 into mitosis (Hunter and Pines, 1995, Cell 80:225-236).

Human tumor suppressors often act as negative regulators of the cell cycle, and several tumor suppressors are known to affect the activities of the CDK/cyclin complexes. For example, p53 activates the transcription of the p21 ($p21^{WAF1/CIP1}$) CDK inhibitor in response to DNA damage signals, and p21 in turn binds and inactivates the CDK4 and CDK6 cyclin D complexes (Gartel et al., 1996, Proc. Soc. Exp. Biol. Med. 213:138-149). Another CDK inhibitor, p16, is itself a potent tumor suppressor (Biggs and Kraft, 1995, J. Mol. Med. 73:509-514). Although multiple members of the p16 and p21 inhibitor families have been identified for other major CDKs, corresponding inhibitors that regulate the mitotic CDK, cdc2, have not previously been identified (Morgan, 1995, Nature 374:131-134).

Cancer Therapy

Currently, cancer therapy may involve surgery, chemotherapy and/or radiation treatment to eradicate neoplastic cells in a patient (see, for example, Stockdale, 1998, "Principles of Cancer Patient Management", in Scientific American: Medicine, vol. 3, Rubenstein and Federman, eds., Chapter 12, Section IV). All of these approaches pose significant drawbacks for the patient. Surgery, for example, may be contraindicated due to the health of the patient or may be unacceptable to the patient. Additionally, surgery may not completely remove the neoplastic tissue. Radiation therapy is only effective when the neoplastic tissue exhibits a higher sensitivity to radiation than normal tissue, and radiation therapy can also often elicit serious side effects.

With respect to chemotherapy, there are a variety of chemotherapeutic agents available for treatment of neoplastic disease. A significant majority of cancer chemotherapeutics act by inhibiting DNA synthesis, either directly, or indirectly by inhibiting the biosynthesis of the deoxyribonucleotide triphosphate precursors, to prevent DNA replication and concomitant cell division (see, for example, Gilman et al., Goodman and Gilman's: The Pharmacological Basis of Therapeutics, Eighth Ed. (Pergamom Press, New York, 1990)). These agents, which include alkylating agents, such as nitrosourea, anti-metabolites, such as methotrexate and hydroxyurea, and other agents, such as etoposides, campathecins, bleomycin, doxorubicin, daunorubicin, etc., although not necessarily cell cycle specific, kill cells during S phase because of their effect on DNA replication. Other agents, specifically colchicine and the vinca alkaloids, such as vinblastine and vincristine, interfere with microtubule assembly resulting in mitotic arrest. Chemotherapy protocols generally involve administration of a combination of chemotherapeutic agents to increase the efficacy of treatment.

Despite the availability of a variety of chemotherapeutic agents, chemotherapy has many drawbacks (see, for example, Stockdale, 1998, "Principles Of Cancer Patient Management" in Scientific American Medicine, vol. 3, Rubenstein and Federman, eds., ch. 12, sect. 10). Almost all chemotherapeutic agents are toxic, and chemotherapy causes significant, and often dangerous, side effects, including severe nausea, bone marrow depression, immunosuppression, etc. Additionally, even with administration of combinations of chemotherapeutic agents, many tumor cells are resistant or develop resistance to the chemotherapeutic agents. In fact, those cells resistant to the particular chemotherapeutic agents used in the treatment protocol often prove to be resistant to other drugs, even those agents that act by mechanisms different from the mechanisms of action of the drugs used in the specific treatment; this phenomenon is termed pleiotropic drug or multidrug resistance. Thus, because of drug resistance, many cancers prove refractory to standard chemotherapeutic treatment protocols. There is a significant need for alternative cancer treatments, particularly for treatment of cancer that has proved refractory to standard cancer treatments, such as surgery, radiation therapy, and chemotherapy.

Pituitary Disorders

The pituitary regulates numerous biological functions through its secretion of different hormones. (For review see Frohman, "The Anterior Pituitary" in Cecil Textbook of Medicine, 18th Ed., Wyngaarden and Smith, eds. (W.B. Saunders Company, Philadelphia, 1988) pp. 1290-1305). In particular, the pituitary releases glycoprotein hormones, which include luteinizing hormone (LH) and follicle stimulating hormone (FSH); LH and FSH regulate ovarian and testicular development as well as reproductive functions such as ovulation and spermatogenesis. Disruption of LH or FSH secretion has dramatic consequences for reproductive function, particularly for ovulation in the female. The pituitary also releases somatomammotropic hormones, including growth hormone and

prolactin. Growth hormone promotes linear growth and is involved in the regulation of certain metabolic functions such as sugar and amino acid uptake and use of fat stores. Prolactin stimulates and maintains lactation in post-parturition females. Although an increase or decrease in prolactin levels does not appear to have significant biological consequences beyond an effect on lactation, disruption of growth hormone secretion stunts growth and has other metabolic effects. Other pituitary hormones include corticotropin (ACTH), thyroid stimulating hormone (TSH), and endorphins and related peptides. Although in some situations, hormone replacement therapy is available, there is a need for additional therapeutics to treat or prevent pituitary dysfunctions.

LATS

The *large tumor suppressor* or *lats* gene (also known as *warts*), a tumor suppressor gene, was previously isolated from *Drosophila* using a mosaic screen. Inactivation of *lats* in somatic cells causes dramatic overproliferation phenotypes (Xu et al., 1995, Development 121:1053-1063; Justice et al., 1995, Genes & Devel. 9:534-546). Somatic cells that are mutant for *lats* undergo extensive proliferation and form large tumors in many tissues of mosaic flies (Xu et al., 1995, Development 121:1053-1063). Tumors that result from inactivation of *lats* display many features of human neoplasms. *Lats* mutant cells grow aggressively, and a single mutant cell can develop into a tumor that is 1/5 the size of the animal, and these fly tumors are highly irregular in shape and size and are often poorly differentiated (St. John and Xu, 1997, Am. J. Hum. Genet 61:1006-1010). *Drosophila* that are homozygous for the various *lats* alleles display a wide range of developmental defects including embryonic lethality, overproliferation of both neural and epidermal tissues, rough eyes, and sterility. Molecular characterization of lats indicates that it contains a putative kinase domain (Xu et al., 1995, Development 121:1053-1063; Justice et al., 1995, Genes & Devel. 9:534-546).

Mouse and human homologs of the *Drosophila lats* have also been identified, and human *lats* was found to be down-regulated in a large number of human tumor cell lines. The nucleotide and amino acid sequences of human lats (h-lats), mouse lats (m-lats), mouse lats2 (m-lats2) and *Drosophila* lats are provided herein in Figures 12-15, respectively (SEQ ID NOS:1-8, respectively), and are described in PCT Publication WO 96/30402, published October 3, 1996, which is incorporated by reference herein in its entirety.

Citation of references hereinabove shall not be construed as an admission that such references are prior art to the present invention.

SUMMARY OF THE INVENTION

The present invention relates to therapeutic and prophylactic methods and compositions for the treatment and prevention of cancers based on lats proteins, and therapeutically and/or prophylactically effective analogs and fragments of lats protein. This is due to the fact that although most tumor suppressor genes regulate the G1/S phase of the

cell cycle, the lats protein interacts with the cell cycle-dependent kinase cdc2, which is involved in the regulation of the G2 to M transition of the cell cycle, and thereby provides a means to regulate the G2 to M transition of the cell cycle and to treat cancers that have proven refractory to other cancer treatments, including chemotherapy and radiation therapy treatments. The invention provides for treatment and prevention of cancer by administration of a therapeutic compound of the invention. The therapeutic compounds of the invention useful for treatment of cancer refractory to a chemotherapy and/or radiation therapy protocol include: lats proteins, and therapeutically effective analogs and derivatives (including fragments) of lats, nucleic acids encoding lats proteins and therapeutically effective analogs and derivatives of lats, and lats agonists.

The invention further provides assays, both *in vivo* and *in vitro*, for testing the efficacy of the therapeutics of the invention for treatment of cancer, particularly cancer that has been shown to be refractory to chemotherapy and radiation therapy treatments.

In another aspect, the invention provides compositions and methods of production of complexes of lats and cdc2 proteins ("lats-cdc2 complexes"), including complexes of lats analogs or derivatives and cdc2 analogs and derivatives (including complexes of lats proteins with cdc2 analogs and derivatives and vice versa), where the analogs and derivatives have the ability to interact with the other member of the complex.

The phosphorylated form of lats complexes with cdc2. Accordingly, in a preferred embodiment, the lats-cdc2 complexes contain phosphorylated lats protein, specifically lats protein phosphorylated on a serine or threonine residue within 20 residues upstream of an Ala-Pro-Glu consensus in subdomain eight of a lats kinase domain, *e.g.*, corresponding to serine 909 of human lats, as depicted in Figure 12 (SEQ ID NO:2). Alternatively, the lats protein in the lats-cdc2 complex has a glutamate or aspartate residue substituted for a serine or threonine residue within 20 residues upstream of an Ala-Pro-Glu consensus in subdomain eight of a lats kinase domain, *e.g.*, corresponding to serine 909 of human lats, as depicted in Figure 12 (SEQ ID NO:2).

In another embodiment, the lats-cdc2 complex contains a portion of lats protein corresponding to amino acids 15-585 of human lats, as depicted in Figure 12 (SEQ ID NO:2).

The invention further provides methods of modulating the activity of cdc2 using lats proteins, as well as lats derivatives and fragments able to interact with cdc2 protein, lats-cdc2 complexes, and antibodies against lats-cdc2 complexes. In particular, the invention provides methods for treating or preventing disorders involving an aberrant level of cdc2 in a subject. Therapeutically effective amounts of compounds are administered to promote or inhibit LATS function, as required.

The invention provides recombinant non-human animals in which a *lats* gene has been inactivated, preferably recombinant mice in which a *lats* gene (preferably a gene having lats coding sequence of SEQ ID NO:3) has been inactivated, i.e., a *lats* knock-out mouse.

In a preferred embodiment, the invention provides a *lats* knock-out mouse in which the inactivated *lats* gene had the coding sequence of SEQ ID NO:3, prior to disruption, and in a more preferred embodiment, the inactivated *lats* gene is deleted for the Lats C-terminal domain 1 (LCD1), the Lats C-terminal domain 2 (LCD2), the Lats C-terminal domain 3 (LCD3), and all or a portion of the kinase domain, and retains the Lats flanking domain (LFD), the Lats split domain 1 (LSD1), the Lats split domain 2 (LSD2), and the putative SH3-binding domain, in a most preferred embodiment the *lats* gene is disrupted by replacement of a non-lats sequence for the sequence encoding the amino acids corresponding to amino acids 756 to 1130 of human lats, as depicted in Figure 12 (SEQ ID NO:2). In other embodiments, the inactivated *lats* gene is deleted for all or a portion of the kinase domain (*e.g.*, so as to inactivate kinase activity). A lats "knock-out" animal is an animal in which at least one genomic copy of a *lats* gene has been inactivated by insertional mutagenesis, *e.g.*, by homologous recombination, for example, as described and exemplified herein.

The invention further provides methods for screening potential therapeutics for activity in the treatment or prevention of cancer, preferably soft tissue sarcomas and ovarian tumors, using the *lats* knock-out animals of the invention. The invention also provides methods for screening potential therapeutics for activity in the treatment or prevention of pituitary dysfunctions, using the *lats* knock-out animals of the invention. In a preferred embodiment, the invention provides methods for screening potential therapeutics for activity in the treatment or prevention of skin cancer using a non-human *lats* knock-out animal, preferably a *lats* knock-out mouse, in which skin tumors have been induced with carcinogens.

Definitions

As used herein, underscoring or italicizing the name of a gene shall indicate the gene, in contrast to its encoded protein product which is indicated by the name of the gene in the absence of any underscoring or italicizing. For example, "*lats*" shall mean the *lats* gene, whereas "lats" shall indicate the protein product of the *lats* gene.

ARN = After Removal of Nocodazole

CDK = Cyclin Dependent Kinase

mlats = mouse lats

BRIEF DESCRIPTION OF THE FIGURES

Figures 1A-H. Human *lats* can functionally replace the fly gene. (A) Adult *Drosophila* in which *lats* homozygous mutant cells have been induced in the imaginal tissues of the *lats* heterozygous larvae, exhibit *lats* mosaic phenotype, and have mutant cells which have undergone extensive proliferation and formed tumors in various body parts of the mosaic adults. (B) Adult *Drosophila* that express human lats (hs-h-lats) completely do not exhibit tumor development (compare to A). (C, D) High magnification views of the

flies in panel (B) show *yellow* bristles (white arrows), indicating lats cells (genetically marked by the *yellow* mutation) were produced and that they have developed into normal structures. (E, F) $lats^{e26\text{-}1}$ homozygous mutants display giant pupae and disc-overproliferation phenotypes (left pupae in panels). Without heat-shock induction, the leaky expression of the *hs-h-lats* transgene partially suppressed the $lats^{e26\text{-}1}$ phenotypes (middle pupae in panels). Daily induction of the *hs-h-lats* gene completely rescued the mutant phenotypes (right pupae in panels). (G) Scanning Election Micrograph view of a *lats* mosaic fly. (H) A *lats* tumor on the wing (indicated by an arrow in panel G) is enlarged, showing that cells in the overproliferated mutant clone have differentiated into wing cells with hair structures.

Figures 2A-E. Phosphorylation of lats oscillates with the cell cycle. (A) The phosphorylation of lats protein in HeLa cells after exposure of the cells to certain conditions was assayed by immunoprecipitation and blotted with an anti-h-lats monoclonal antibody. "CIP" indicates that the cells were incubated in calf intestinal phosphatase and "ß-gp" indicates that the cells were incubated in ß-glycerol phosphate. The "Time (min.)" indicates time in minutes of incubation. "℗-h-Lats" and "h-Lats" indicate the phosphorylated and dephosphorylated forms of h-lats, respectively. Lats proteins from mitotic HeLa cell lysates (50 min. ARN (After Removal of Nocodazole)) display a slow-migrating form on SDS-PAGE (6%) (lane 1). The proteins are converted into a fast-migrating form when incubated with Calf Intestinal Phosphatase ("CIP") (lanes 2-4). When both CIP and a CIP inhibitor, ß-glycerol phosphate ("ß-gp"), are present, their mobility remains unchanged (lane 5). Lats proteins from 125 min ARN cells have both the slow-migrating and fast-migrating forms (lane 6) and CIP-treatment converts all lats proteins into the fast-migrating form (lanes 7-9). (B) Immunowestern blot shows that phosphorylation of the lats protein oscillates with the cell cycle. Cell cycle stages G0, G1, S, and G2, are indicated above each lane and cells in different mitotic stages (M) are indicated by min. (minutes ARN). The faint bands are degradation products of lats. The progression of the cell cycle was verified by DAPI staining. (C-E) These panels show fluorescent micrographs of DAPI staining for cells at three time points (50' (C), 75' (D), and 100' (E) ARN). Arrows indicate cells at metaphase (50' ARN (panel C)), anaphase (75' ARN (panel D)), or telophase (100' ARN (panel E)), respectively.

Figures 3A-E. Lats directly complexes with cdc2 during mitosis and the lats/cdc2 complex is inactive for H1 kinase activity. (A) Cdc2 is co-immunoprecipitated with lats from mitotic CHO cell lysates (M) but not from quiescent CHO cell lysates (G0). Anti-h-lats polyclonal antibodies or anti-human cyclin B monoclonal antibodies were used for immunoprecipitation, and anti-human cdc2 monoclonal antibodies were used to visualize cdc2. (B) Cdc2 co-immunoprecipitated with human lats proteins at early mitosis. The stages of the cell cycle are indicated above each lane as "min. ARN" or "G0". Lats proteins were immunoprecipitated using anti-h-lats monoclonal antibodies and were separated on 8% SDS-PAGE. The western blot was sequentially probed with anti-human cdc2

monoclonal antibodies (lower panel, labeled "Cdc2"), anti-h-lats monoclonal antibodies (upper panel, labeled "h-lats"), anti-human cyclin B and cyclin A monoclonal antibodies (middle panels, labeled "Cyclin B" and "Cyclin A", respectively). (C) Co-immunoprecipitation of baculovirus-expressed human lats and cdc2 proteins. H-lats proteins were precipitated with anti-h-lats monoclonal antibodies and probed with anti-human cdc2 monoclonal antibodies (upper panel, labeled "Cdc 2") and cdc2 proteins were precipitated with anti-human cdc2 monoclonal antibodies and probed with anti-h-lats monoclonal antibodies (lower panel, labeled "h-lats"). (D) Lats-associated cdc2 is inactive for H1 kinase activity. Cdc2 proteins co-immunoprecipitated with either lats or cyclin B (indicated by "+" in the legend labeled "anti-Cyclin B" or "anti-h-lats") from 50 min. ARN HeLa cell lysates were divided for western quantification of cdc2 (upper panel, labeled "IP-Western") and for the histone H1 kinase assay (lower panel, labeled "H1 Kinase assay"), respectively. As a control, Protein G-agarose beads incubated with equal amounts of cell lysates only were also used for the H1 kinase assay (indicated by "-" in the legend). (E) Summarized are the results of yeast two-hybrid assays for interactions among full-length h-lats (h-lats), N-terminal region of h-lats (N-h-lats), C-terminal region of h-lats (C-h-lats), human cdc2, CDK2, and CDK4. +++, ++, +: indicate strong, intermediate, and weak interactions, respectively, while - and ND indicate no interaction and not determined, respectively.

Figures 4A-F. Genetic interaction between *lats*, *cdc2*, and *cyclin A* in *Drosophila*. (A) $lats^{P8}/lats^{P8}$ homozygotes die at the pupal stage. (B) Removal of one copy of the *cdc2* gene rescues $lats^{P8}$ lethality ($lats^{P8}/lats^{P8}$; $+/cdc2^{B47}$). (C) A typical rough, overproliferated eye dissected from a $lats^{P8}/lats^{P8}$ dead pupa. (D) An eye from the fly in panel (B), showing that the eye phenotype has been almost completely suppressed. (E) $lats^{e26\text{-}1}/lats^{e26\text{-}1}$ pupae (the pupa labeled 1) are much larger than wild-type (the pupa labeled 4). The giant-pupa phenotype is partially reduced by a *cdc2* temperature sensitive mutant at room temperature (pupa labeled 2) ($lats^{e26\text{-}1}/lats^{e26\text{-}1}$; $cdc2^{E1\text{-}24}/cdc2^{E1\text{-}24}$) or by removal of one copy of the *cdc2* gene ([i[a labeled 3) ($lats^{e26\text{-}1}/lats^{e26\text{-}1}$; $+/cdc2^{B47}$). (F) The third instar larval wing discs dissected from animals of the same genotypes in (E) and labeled with the same number. The lats disc overproliferation phenotype (larva 1) is dramatically suppressed by mutations in the *cdc2* gene (larvae 2 and 3). Removal of one copy of the *cyclin A* gene resulted in a phenotypic suppression of the *lats* mutants in a manner similar to removal of one *cdc2* gene as shown above.

Figures 5A-N. Effect of inactivation and overexpression of lats on the cell cycle in *Drosophila*. (A) *Drosophila* third instar eye imaginal disc contains a homozygous $lats^{x1}$ clone (arrowhead indicates the lack of Myc staining) that crosses the morphogenetic furrow (MF) (arrow). (B) Cyclin A staining (indicated by arrowhead) in the clone exhibits expression that spans the MF (arrow). (C) Composite staining of the same disc shown in panels A and B showing Myc stains and propidium iodide staining which more clearly delineates the MF and the *lats* mutant clone (indicated by the arrow) that spans it. (D)

Composite staining magnification of the same *lats* mutant clone shown in panels A-C. Cyclin A staining clearly spans the MF region (arrow). (E) Composite staining of the same region shown in panel D viewed with Myc and propidium iodide. (F) Composite staining magnification of the same clone shown in panel E viewed with Cyclin A staining and propidium iodide. Cyclin A is degraded in *lats* mutant cells at late mitosis (as indicated by the arrowheads). (G) *Drosophila* third instar eye disc containing homozygous $lats^{x1}$ clones (lack of Myc staining is indicated by arrowheads) that span the MF region. (H) The third instar eye disc shown in panel G was also stained for Cyclin B. The MF (indicated by the arrow) is well defined and free of cyclin B staining. (I and J) Scanning electron micrograph of a wild-type *Drosophila* adult eye (I) in comparison to an eye (J) of a *GMR-d-lats* transgenic fly showing fewer and irregular ommatidia and missing bristles. (K) A section of a *GMR-d-lats* eye reveals that many pigment cells are missing, and that ommatidia occasionally lack a full complement of photoreceptor cells (arrow). (L) Propidium iodide staining of a *GMR-d-lats* third instar eye imaginal disc. A stripe of intensely stained nuclei which are tetraploid (indicated by the star) are seen in the region of the second mitotic wave, which is immediately followed posteriorly by apoptotic cells with fragmented nuclei (indicated by the small arrows). (M) BrdU labeling (green) of a *GMR-d-lats* third instar eye disc reveals that S phase occurs in the second mitotic wave (indicated by the star) posterior to the MF (indicated by the arrow) just as it would in wild type. (N) A *GMR-p21* eye disc is shown in which cell proliferation in the second mitotic wave is blocked before S phase and the stripe of BrdU labeling posterior to the MF (indicated by the arrow) is abolished.

Figures 6A-D. Targeting of the *lats* locus by homologous recombination. (A) Sequence alignment of human *lats* (h-*lats*) and mouse *lats* (m-*lats*, partial sequence). Arrow indicates the point at which the mouse *lats* gene was disrupted. (B) Targeting vector for positive-negative selection of homologous recombinants at the lats locus, with restriction map and the structure of the targeted lats locus. The vector is represented by the second line from the top, while the wild-type and mutant (i.e., disrupted) lats alleles are indicated by the top and bottom lines, respectively. The BamHI sites are indicated by "B", the EcoRI sites are indicated by "R", and the EcoRV sites are indicated by "RV". Exons are represented by filled rectangles. A BamHI/EcoRV double digest generates a 3.5 kb fragment from the wild-type allele and a 5.8 kb fragment from the disrupted allele, both of which are recognized by the probe shown, which is not contained in the targeting vector. In the vector and the mutant allele, the PGK-TK gene cassette and the PGK-neo fragment are denoted by open boxes labeled accordingly. (C) Southern blot of genomic DNA isolated from individual embryonic stem cell clones. The genotypes of the clones are indicated above the lanes with the "+/+" indicating wild-type clones, "+/-" indicating clones heterozygous for the mutant allele, and "-/-" indicating clones homozygous for the mutant allele. (D) Western blot using anti-h-lats polyclonal antibody on lysates from 13.5 dpc (days post coitus) mouse embryonic fibroblasts indicating the absence of lats protein in the knock-out mice. The genotype of the clones is indicated above the lanes as in panel C.

Figures 7A and B. Growth retardation of *lats*$^{-/-}$ mice. (A) Representative picture of a *lats*$^{-/-}$ mouse (agouti, the mouse on the right) with its wild-type littermate (black, the mouse on the left) (12-days-old). (B) Representative growth curve of *lats*$^{+/+}$, *lats*$^{+/-}$, and *lats*$^{-/-}$ mice. Mice were weighed (grams (g)) at intervals and plotted against age in days.

Figures 8A-D. Ovarian phenotypes of *lats*$^{-/-}$ mice. Histopathological sections of ovaries derived from *lats*$^{+/+}$ (panels A and C) and *lats*$^{-/-}$ (panels B and D) females. Overview images are in panels A and B while high magnification views are in panels C and D. The paraffin sections were stained with hematoxylin and eosin. An absence of corpora lutea (CL) is evident in the *lats*$^{-/-}$ ovary. Ovarian stromal cell tumors (SC) which obliterate the normal structure of the ovary, eliminating follicles (FC) progressively, are readily apparent.

Figures 9A-F. Absence of mammary gland development in *lats*$^{-/-}$ Mice. (A,B) *Lats*$^{-/-}$ female with normal mammary gland and nipple development. (D,E) *Lats*$^{-/-}$ female displaying absence of mammary gland and nipple formation. (C,F) Hematoxylin and eosin stained histopathological sections of mammary glands derived from *lats*$^{+/+}$ (C) and *lats*$^{-/-}$ (F) mice. The amount of breast epithelial tissue was markedly decreased in *lats*$^{-/-}$ females, resulting in mammary fat pads, devoid of an epithelial component.

Figures 10A-E. Pituitary hyperplasia and dysfunction in *lats*$^{-/-}$ mice. (A and B) Histopathological sections of pituitaries derived from lats$^{+/+}$ (A) and *lats*$^{-/-}$ (B) mice. The paraffin sections were stained with hematoxylin and eosin. The normal pituitary gland from a *lats*$^{+/+}$ mouse demonstrates the organized architecture of the gland. Hyperplastic changes are visible in *lats*$^{-/-}$ pituitaries. Multiple atypical cells showing irregularly shaped nuclei, and variability in shape and size are readily apparent. (C-E) The graphs indicate the amount of LH (C), PRL (D), and FSH (E) production in the mice. Hormone levels are plotted as ng/ml with the results from the *lats*$^{+/-}$ mice represented by the right bar of each bar graph and the results from the *lats*$^{-/-}$ mice represented by the left bar of each bar graph.

Figures 11A-C. Soft tissue sarcoma development in *lats*$^{-/-}$ mice. (A,B) Typical soft tissue sarcomas in *lats*$^{-/-}$ mice (A, 6.5 months old; B, 4.5 months old). (C) Histopathological section of the soft tissue sarcoma shown in panel B stained with hematoxylin and eosin revealing pleiomorphic, spindle-shaped cells characteristic of this tumor.

Figure 12. Nucleotide and amino acid sequences of human lats (h-lats) (SEQ ID NOS:1 and 2, respectively).

Figure 13. Nucleotide and amino acid sequences of mouse lats (m-lats) (SEQ ID NOS:3 and 4, respectively).

Figure 14. Nucleotide and amino acid sequences of mouse lats2 (m-lats2) (SEQ ID NOS:5 and 6, respectively).

Figure 15. Nucleotide and amino acid sequences of *Drosophila* lats (SEQ ID NOS:7 and 8, respectively).

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides lats-cdc2 protein complexes, including complexes that contain lats analogs and fragments and/or cdc2 analogs and fragments, as well as methods of producing these complexes and nucleic acids encoding the two members of the complex. The invention also provides antibodies that bind immunospecifically to a lats-cdc2 complex, but do not bind the individual binding partners immunospecifically.

The invention also provides methods for the modulation of cdc2 activity using lats proteins and lats analogs and derivatives that are able to interact with cdc2. In particular, methods are provided for treating or preventing diseases and disorders associated with aberrant cdc2 activity by administration of a therapeutic compound of the invention. The present invention further provides recombinant non-human animals, preferably mice, having at least one copy of (preferably both copies of, *i.e.*, is homozygous for) an inactivated *lats* gene, *i.e.*, *lats* knock-out animals. Preferably, these *lats* knock-out animals are generated by homologous recombination, *i.e.*, have a gene disrupted by insertional mutagenesis induced by homologous recombination with a nucleic acid containing non-lats sequences flanked by *lats* genomic sequences. The invention further provides methods of screening for compounds effective to treat or prevent cancer, preferably soft tissue sarcomas or ovarian tumors, more preferably skin cancer, using the recombinant non-human animals of the invention. The invention also provides methods of screening for compounds effective to treat or prevent pituitary dysfunction using the recombinant non-human animals of the invention.

Therapeutics of the invention that can be used to treat or prevent diseases and disorders associated with an aberrant level of cdc activity include those therapeutics that promote lats function (*e.g.*, lats proteins and lats derivatives and analogs that supply lats function, nucleic acids encoding lats, lats derivatives and analogs, and lats-cdc2 complexes), and those therapeutics that inhibit or antagonize lats function (*e.g.*, lats derivatives and analogs that inhibit or antagonize lats function) and nucleic acids encoding these lats derivatives and analogs, anti-lats antibodies and anti-lats-cdc2 complex antibodies, lats antisense nucleic acids, and lats inhibitors and antagonists.

The present invention also provides therapeutic methods and compositions for the treatment and prevention of cancer based on lats proteins and therapeutically or prophylactically effective analogs and fragments of lats proteins. The invention provides for treatment and prevention of cancer by administration of a therapeutic compound of the invention. The therapeutic compounds of the invention that can be used to treat or prevent cancer include: lats proteins, including human lats proteins, therapeutically or prophylactically effective lats analogs and fragments, and nucleic acids encoding the lats proteins, analogs and fragments.

In a preferred embodiment, the invention provides therapeutic and prophylactic methods for the treatment or prevention of cancer that has been shown to be or may be

refractory to chemotherapy or radiation therapy treatments or treatments based on tumor suppressor genes other than lats.

Also included in the invention are methods of screening lats proteins and lats derivatives and analogs for activity in treating cancer that has been shown to be or may be refractory to chemotherapy or radiation therapy. Additionally, the invention provides methods of screening lats proteins, lats derivatives and fragments, anti-lats antibodies, lats antisense nucleic acids, lats antagonists and inhibitors, and lats-cdc2 complexes for activity in modulating the activity of cdc2.

Therapeutic Uses

The invention provides for treatment or prevention of cancers refractory to chemotherapy or radiation therapy by administration of a therapeutic compound (termed herein "Therapeutic"). The invention also provides for treatment or prevention of diseases or disorders that can be treated by modulation of cdc2 activity by administration of a Therapeutic of the invention. Such "Therapeutics" include lats proteins and therapeutically or prophylactically effective analogs and fragments thereof; lats-cdc2 complexes; antibodies thereto; nucleic acids encoding the lats proteins, analogs, or fragments, and lats-cdc2 complexes; lats antisense nucleic acids, and lats agonists and antagonists .

In specific embodiments, the therapeutic is a lats protein or lats-cdc2 complex containing a lats protein that is phosphorylated, particularly a lats protein phosphorylated on a serine or threonine residue within 20 residues upstream of an Ala-Pro-Glu consensus in subdomain eight of a lats kinase domain, *e.g.*, a serine corresponding to serine 909 of human lats, as depicted in Figure 12 (SEQ ID NO:2). In another embodiment, the therapeutic is a lats derivative or lats-cdc2 complex containing a lats derivative, in which derivative a serine or threonine residue within 20 residues upstream of an Ala-Pro-Glu consensus in subdomain eight of a lats kinase domain is substituted with a glutamate or aspartate residue, preferably the serine corresponding to serine 909 of human lats is replaced with a glutamate residue. In a further embodiment, the therapeutic is a fragment of a lats protein or a lats-cdc2 complex containing a fragment of a lats protein comprising or consisting of the amino acid sequence of a lats protein corresponding to amino acids 15-585 of human lats, as depicted in Figure 12 (SEQ ID NO:2).

Generally, administration of products of a species origin or species reactivity (in the case of antibodies) that is the same species as that of the subject is preferred. Thus, in a preferred embodiment, a human lats protein, derivative, or fragment, or nucleic acid, or an antibody to a human lats protein, is therapeutically or prophylactically administered to a human patient.

Treatment and Prevention of Cancers Refractory to Chemotherapy or Radiotherapy

Cancers, including neoplasms, tumors, metastases, or any disorder characterized by uncontrolled cell growth, that have been shown to be refractory to a chemotherapy or

radiation therapy can be treated or prevented by administration of a Therapeutic of the invention that promotes (*i.e.*, increases or supplies) lats function.

Examples of such a Therapeutic include lats proteins, derivatives or fragments that are functionally active, particularly have a lats functional activity of inhibiting cell overproliferation (*e.g.*, as demonstrated in *in vitro* assays or in an animal model), and nucleic acids encoding a lats protein or a functionally active derivative or analog thereof (*e.g.*, for use in gene therapy). Other Therapeutics that can be used, *e.g.*, lats agonists, can be identified using *in vitro* assays or animal models, examples of which are described in Examples section.

That a cancer is refractory to chemotherapy or radiation therapy means that at least some significant portion of the cancer cells are not killed or their cell division arrested by the particular chemotherapeutic agent or combination of chemotherapeutic agents or the level of radiation employed in a therapeutic protocol. The determination of whether the cancer cells are refractory to the chemotherapy or the radiation therapy can be made either *in vivo* or *in vitro* by any method known in the art for assaying the effectiveness of treatment on cancer cells.

In various embodiments of the invention, cancer that is refractory to radiation therapy, chemotherapy or combination chemotherapy, or combination of radiotherapy and chemotherapy, is treated or prevented by administration of a Therapeutic of the invention. In a preferred embodiment, cancer that is refractory to treatment with a chemotherapeutic agent that is cell cycle specific, or said cancer is refractory to treatment with a chemotherapeutic agent that kills or arrests the cells in the S phase of the cell cycle, or said cancer is refractory to treatment with a chemotherapeutic agent that kills or arrests cells during the M phase of the cell cycle is treated using a Therapeutic of the invention. The Therapeutic of the invention can be administered along with radiation therapy and/or one or a combination of chemotherapeutic agents, or as an alternative to other forms of therapy.

The chemotherapy or radiation therapy administered concurrently with or subsequent to the administration of the therapeutic of the invention can be administered by any method known in the art. The chemotherapeutic agents are preferably administered in a series of sessions, any one or a combination of the chemotherapeutic agents listed above can be administered. With respect to radiation therapy, any radiation therapy protocol can be used depending upon the type of cancer to be treated. For example, but not by way of limitation, x-ray radiation can be administered; in particular, high-energy megavoltage (radiation of greater that 1 MeV energy) can be used for deep tumors, and electron beam and orthovoltage x-ray radiation can be used for skin cancers. Gamma ray emitting radioisotopes, such as radioactive isotopes of radium, cobalt and other elements may also be administered to expose tissues to radiation.

Malignancies

Malignancies and related disorders that may become refractory to chemotherapy and/or radiation therapy and that can be treated or prevented by administration of a Therapeutic that promotes lats function include blood-related cancers and solid tumors (for a review of such disorders, see Fishman et al., 1985, Medicine, 2d Ed., J.B. Lippincott Co., Philadelphia).

Treatment and Prevention of Diseases by Modulation of Cdc2 Activity

In a specific embodiment of the invention, diseases and disorders associated with aberrant levels of cdc2 activity, *e.g.*, aberrantly high or aberrantly low levels of cdc2 protein or activity, can be treated or prevented by administration of a therapeutic of the invention able to modulate the activity of cdc2. In particular, those diseases and disorders associated with an aberrantly high cdc2 activity are treated or prevented by administration of a Therapeutic that promotes lats activity. Alternatively, those diseases and disorders associated with an aberrantly low cdc2 activity are treated or prevented by administration of a Therapeutic that inhibits lats activity, *e.g.*, lats derivatives and analogs that inhibit or antagonize lats activity, anti-lats antibodies, lats antisense nucleic acids, lats inhibitors and antagonists, antibodies that specifically recognize a lats-cdc2 complex, etc.

Because cdc2 promotes cell division, diseases and disorders that may be associated with an increased level of cdc2 activity, as compared with the levels of cdc2 in a subject not afflicted with such a disease or disorder, include diseases and disorders associated with increased cell proliferation, such as malignancies. Diseases and disorders that may be associated with a decreased level of cdc2 activity include diseases and disorders associated with decreased cell proliferation.

Premalignant Conditions

The Therapeutics of the invention that reduce cdc2 activity can be administered to treat premalignant conditions and to prevent progression to a neoplastic or malignant state. Such prophylactic or therapeutic use is indicated in conditions known or suspected of preceding progression to neoplasia or cancer, in particular, where non-neoplastic cell growth consisting of hyperplasia, metaplasia, or most particularly, dysplasia has occurred.

Alternatively or in addition to the presence of abnormal cell growth characterized as hyperplasia, metaplasia, or dysplasia, the presence of one or more characteristics of a transformed phenotype, or of a malignant phenotype, displayed *in vivo* or displayed *in vitro* by a cell sample from a patient, can indicate the desirability of prophylactic/therapeutic administration of a Therapeutic that inhibits cdc2 activity. Some characteristics of a transformed phenotype include morphology changes, looser substratum attachment, loss of contact inhibition, loss of anchorage dependence, protease release, increased sugar transport, decreased serum requirement, expression of fetal antigens, disappearance of the 250,000 dalton cell surface protein, etc.

Treatment and Prevention of Disorders in Which Cell Proliferation Is Desired

Diseases and disorders involving a deficiency in cell proliferation (growth) or in which cell proliferation is otherwise desirable for treatment or prevention, are treated or prevented by administration of a Therapeutic that antagonizes (inhibits) lats function (in particular, lats-mediated inhibition of cell proliferation and/or lats binding to cdc2). Therapeutics that can be used include anti-lats antibodies (and fragments and derivatives thereof containing the binding region thereof), lats derivatives or fragments that are dominant-negative kinases, *lats* antisense nucleic acids, and *lats* nucleic acids that are dysfunctional (*e.g.*, due to a heterologous (non-lats sequence) insertion within the lats coding sequence) that are used to "knockout" endogenous lats function by homologous recombination (see, *e.g.*, Capecchi, 1989, Science 244:1288-1292), as described herein. Other Therapeutics that inhibit lats function can be identified by use of known convenient *in vitro* assays, *e.g.*, based on their ability to inhibit binding of lats to another protein (*e.g.*, cdc2), or inhibit any known lats function, as preferably assayed *in vitro* or in cell culture. Methods for screening for compounds that prevent or reduce lats binding to cdc2 are described herein. Preferably, suitable *in vitro* or *in vivo* assays are utilized to determine the effect of a specific Therapeutic (*i.e.*, its ability to promote cdc2 activity or increase cdc2 levels) and whether its administration is indicated for treatment of the affected tissue.

Diseases and disorders involving a deficiency in cell proliferation or in which cell proliferation is desired for treatment or prevention, and that can be treated or prevented by promoting cdc2 function, include degenerative disorders, growth deficiencies, hypoproliferative disorders, physical trauma, lesions, and wounds; for example, to promote wound healing, or to promote regeneration in degenerated, lesioned or injured tissues, etc.

Gene Therapy

Gene therapy refers to therapy performed by the administration of a nucleic acid to a subject. In this embodiment of the invention, the nucleic acid produces its encoded protein that mediates a therapeutic effect by promoting lats function.

Any of the methods for gene therapy available in the art can be used according to the present invention. Exemplary methods are described below.

For general reviews of the methods of gene therapy, see Goldspiel et al., 1993, Clinical Pharmacy 12:488-505; Wu and Wu, 1991, Biotherapy 3:87-95; Tolstoshev, 1993, Ann. Rev. Pharmacol. Toxicol. 32:573-596; Mulligan, 1993, Science 260:926-932; and Morgan and Anderson, 1993, Ann. Rev. Biochem. 62:191-217; May, 1993, TIBTECH 11(5):155-215. Methods commonly known in the art of recombinant DNA technology which can be used are described in Ausubel et al. (eds.), 1993, Current Protocols in Molecular Biology, John Wiley & Sons, NY; and Kriegler, 1990, Gene Transfer and Expression, A Laboratory Manual, Stockton Press, NY.

In a preferred aspect, the Therapeutic comprises a *lats* nucleic acid that is part of an expression vector that expresses a lats protein or fragment or chimeric protein thereof in a

suitable host. In particular, such a nucleic acid has a promoter operably linked to the lats coding region, said promoter being inducible or constitutive, homologous or heterologous, and, optionally, tissue-specific. In another particular embodiment, a nucleic acid molecule is used in which the lats coding sequences and any other desired sequences are flanked by regions that promote homologous recombination at a desired site in the genome, thus providing for intrachromosomal expression of the *lats* nucleic acid, as described (Koller and Smithies, 1989, Proc. Natl. Acad. Sci. USA 86:8932-8935; Zijlstra et al., 1989, Nature 342:435-438). In another embodiment, a nucleic acid or combination of nucleic acids containing both a *lats* and a *cdc2* nucleic acid, preferably where each is operably linked to a promoter, is delivered by gene therapy methods.

Delivery of the nucleic acid into a patient may be either direct, in which case the patient is directly exposed to the nucleic acid or nucleic acid-carrying vector, or indirect, in which case, cells are first transformed with the nucleic acid *in vitro*, then transplanted into the patient. These two approaches are known, respectively, as *in vivo* or *ex vivo* gene therapy.

Numerous techniques are known in the art for the introduction of foreign genes into cells (see *e.g.*, Loeffler and Behr, 1993, Meth. Enzymol. 217:599-618; Cohen et al., 1993, Meth. Enzymol. 217:618-644; Cline, 1985, Pharmac. Ther. 29:69-92).

In an embodiment in which recombinant cells are used in gene therapy, a *lats* nucleic acid or both *lats* and *cdc2* nucleic acids are introduced into the cells such that they are expressible by the cells or their progeny, and the recombinant cells are then administered *in vivo* for therapeutic effect. In a specific embodiment, stem or progenitor cells are used. Any stem and/or progenitor cells which can be isolated and maintained *in vitro* can potentially be used, such as hematopoietic stem cells (HSC), stem cells of epithelial tissues such as the skin and the lining of the gut, embryonic heart muscle cells, liver stem cells (PCT Publication WO 94/08598, dated April 28, 1994), neural stem cells (Stemple and Anderson, 1992, Cell 71:973-985), or epithelial stem cells (ESCs) (Rheinwald, 1980, Meth. Cell Bio. 21A:229; Pittelkow and Scott, 1986, Mayo Clinic Proc. 61:771).

Antisense Therapy

Lats function may be inhibited by use of *lats* antisense nucleic acids. The present invention provides the therapeutic or prophylactic use of nucleic acids of at least six nucleotides and are preferably oligonucleotides (ranging from 6 to about 200 oligonucleotides), that are antisense to a gene or cDNA encoding a lats protein, or portions thereof. A *lats* "antisense" nucleic acid as used herein refers to a nucleic acid capable of hybridizing to a portion of a *lats* nucleic acid (preferably mRNA) by virtue of some sequence complementarity. The antisense nucleic acid may be complementary to a coding and/or noncoding region of a *lats* mRNA. In specific aspects, the oligonucleotide is at least 10 nucleotides, at least 15 nucleotides, at least 100 nucleotides, or at least 200 nucleotides.

The oligonucleotides can be DNA or RNA or chimeric mixtures or derivatives or modified versions thereof, single-stranded or double-stranded. The oligonucleotide can be modified at any position (examples of such modifications can be found in: Bailey, Ullmann's Encyclopedia of Industrial Chemistry (1998), 6th ed. Wiley and Sons). Such antisense nucleic acids have utility as Therapeutics that inhibit lats function or activity, and can be used in the treatment or prevention of disorders characterized by an aberrantly low cdc2 level or activity.

The *lats* antisense nucleic acids can be directly administered to a cell, or can be produced intracellularly by transcription of exogenous, introduced sequences. Alternatively, *lats* antisense nucleic acids are produced intracellularly by transcription from an exogenous sequence. For example, a vector can be introduced *in vivo* such that it is taken up by a cell, within which cell the vector or a portion thereof is transcribed, producing an antisense nucleic acid (RNA) of the invention. Such a vector can remain episomal or become chromosomally integrated, as long as it can be transcribed to produce the desired antisense RNA. Such vectors can be constructed by recombinant DNA technology methods standard in the art.

The antisense nucleic acids of the invention comprise a sequence complementary to at least a portion of an RNA transcript of a *lats* gene, preferably a human *lats* gene. However, absolute complementarity, although preferred, is not required.

Pharmaceutical compositions of the invention, comprising an effective amount of a *lats* antisense nucleic acid in a pharmaceutically acceptable carrier can be administered to a patient having a disease or disorder which is characterized by aberrantly low cdc2 activity.

The amount of *lats* antisense nucleic acid that will be effective in the treatment of a particular disorder or condition will depend on the nature of the disorder or condition, and can be determined by standard clinical techniques. Where possible, it is desirable to determine the antisense cytotoxicity *in vitro*, and then in useful animal model systems prior to testing and use in humans.

In a specific embodiment, pharmaceutical compositions comprising *lats* antisense nucleic acids are administered via liposomes, microparticles, or microcapsules. In various embodiments of the invention, it may be useful to use such compositions to achieve sustained release of the *lats* antisense nucleic acids. In a specific embodiment, it may be desirable to utilize liposomes targeted via antibodies to specific identifiable central nervous system cell types (Leonetti et al., 1990, Proc. Natl. Acad. Sci. U.S.A. 87: 2448-2451; Renneisen et al., 1990, J. Biol. Chem. 265: 16337-16342).

Lats Proteins, Derivatives, Fragments and Lats-cdc2 Complexes

The lats proteins and nucleic acids, and lats derivatives and fragments can be produced by any method known in the art.

For recombinant expression of lats proteins, and lats derivatives and fragments, the nucleic acid containing all or a portion of the nucleotide sequence encoding the protein can

be inserted into an appropriate expression vector, *i.e.*, a vector that contains the necessary elements for the transcription and translation of the inserted protein coding sequence. In a preferred embodiment, the regulatory elements (*e.g.*, promoter) are heterologous (*i.e.*, not the native gene promoter). Promoters which may be used include the SV40 early promoter (Bernoist and Chambon, 1981, Nature 290: 304-310), and the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (Yamamoto et al., 1980, Cell 22: 787-797), among others.

A variety of host-vector systems may be utilized to express the protein coding sequence. These include mammalian cell systems infected with virus (*e.g.*, vaccinia virus, adenovirus, etc.); insect cell systems infected with virus (*e.g.* baculovirus); microorganisms such as yeast containing yeast vectors; or bacteria transformed with bacteriophage, DNA, plasmid DNA, or cosmid DNA.

Once a lats protein, or derivative or fragment, has been recombinantly expressed, it may be isolated and purified by standard methods including chromatography (*e.g.*, ion exchange, affinity, and sizing column chromatography), centrifugation, differential solubility, or by any other standard technique for the purification of proteins. A lats protein may also be purified by any standard purification method from natural sources.

Alternatively, a lats protein, analog or derivative can be synthesized by standard chemical methods known in the art (*e.g.*, see Hunkapiller et al., 1984, Nature 310:105-111).

The Therapeutics of the invention also include derivatives and fragments related to lats. In particular embodiments, the derivative or fragment is functionally active, *i.e.*, capable of exhibiting one or more functional activities associated with a full-length, wild-type lats protein, *e.g.*, able to inhibit cell proliferation in *in vitro* and/or *in vivo* assays. Additionally, derivatives or fragments that inhibit lats activity, *e.g.*, promote cell proliferation, may also have a use in the methods of the invention. Derivatives or analogs of lats can be tested for the desired activity by procedures known in the art.

In specific embodiments of the invention, the Therapeutic is a lats protein that is phosphorylated, preferably that is phosphorylated on a serine or threonine residue within 20 amino acids upstream of an Ala-Pro-Glu consensus sequence in subdomain eight of a lats kinase domain, more preferably that is phosphorylated on a serine residue corresponding to serine 909 of human lats, as depicted in Figure 12 (SEQ ID NO:2). In another specific embodiment, the therapeutic is a lats derivative in which a serine or threonine residue within 20 residues upstream of an Ala-Pro-Glu consensus in subdomain eight of a lats kinase domain is substituted with a glutamate or aspartate residue, preferably, in which a serine residue corresponding to serine 909 of human lats is replaced with a glutamate residue. In another specific embodiment, the therapeutic is a fragment of a lats protein comprising or consisting of the amino acid sequence corresponding to amino acids 15 to 585 of human lats, as depicted in Figure 12 (SEQ ID NO:2).

In particular, lats derivatives can be made by altering lats sequences by substitutions, additions or deletions that provide for functionally equivalent molecules. Due

to the degeneracy of nucleotide coding sequences, other DNA sequences which encode substantially the same amino acid sequence as a *lats* gene may be used in the practice of the present invention. These include nucleotide sequences comprising all or portions of *lats* genes which are altered by the substitution of different codons that encode a functionally equivalent amino acid residue within the sequence, thus producing a silent change. Likewise, the lats derivatives of the invention include those containing, as a primary amino acid sequence, all or part of the amino acid sequence of a lats protein including altered sequences in which functionally equivalent amino acid residues are substituted for residues within the sequence resulting in a silent change. For example, one or more amino acid residues within the sequence can be substituted by another amino acid of a similar polarity which acts as a functional equivalent, resulting in a silent alteration. Substitutes for an amino acid within the sequence may be selected from other members of the class to which the amino acid belongs. For example, the nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan and methionine. The polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine. The positively charged (basic) amino acids include arginine, lysine and histidine. The negatively charged (acidic) amino acids include aspartic acid and glutamic acid.

In a specific embodiment of the invention, proteins consisting of or comprising a fragment of a lats protein consisting of at least 10 (continuous) amino acids of the lats protein is provided. In other embodiments, the fragment consists of at least 20 or 50 amino acids of the lats protein. In specific embodiments, such fragments are not larger than 35, 100 or 200 amino acids. In a specific embodiment, the fragment of a lats protein is from the N-terminal portion of the protein, preferably including all or a portion of the amino acids corresponding to amino acids 15-585 of human lats. Derivatives or fragments of lats include but are not limited to those molecules comprising regions that are substantially homologous to lats or fragments thereof (*e.g.*, in various embodiments, at least 60% or 70% or 80% or 90% or 95% identity over an amino acid sequence of identical size with no insertions or deletions considered, or when compared to an aligned sequence in which the alignment is done by a computer homology program known in the art, *e.g.*, the blastp program) or whose encoding nucleic acid is capable of hybridizing to the inverse complement (the inverse complement of a nucleic acid strand has the complementary sequence running in reverse orientation to the strand so that the inverse complement would hybridize without mismatches to the nucleic acid strand; thus, for example, where the coding strand is hybridizable to a nucleic acid with no mismatches between the coding strand and the hybridizable strand, then the inverse complement of the hybridizable strand is identical to the coding strand) of a coding lats sequence, under high stringency, moderately stringency, or low stringency conditions, as discussed *infra*.

The lats derivatives and fragments of the invention can be produced by various methods known in the art. The manipulations which result in their production can occur at

the gene or protein level. For example, the cloned *lats* gene sequence can be modified by any of numerous strategies known in the art (Sambrook et al., 1990, Molecular Cloning, A Laboratory Manual, 2d ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, New York).

Additionally, the lats-encoding nucleic acid sequence can be mutated *in vitro* or *in vivo*, to create and/or destroy translation, initiation, and/or termination sequences, or to create variations in coding regions and/or form new restriction endonuclease sites or destroy preexisting ones, to facilitate further *in vitro* modification. Any technique for mutagenesis known in the art can be used, including chemical mutagenesis, *in vitro* site-directed mutagenesis (Hutchinson, C., et al., 1978, J. Biol. Chem 253:6551), use of TAB® linkers (Pharmacia), etc.

Manipulations of the lats sequence may also be made at the protein level. Included within the scope of the invention are lats protein fragments or other derivatives which are differentially modified during or after translation, *e.g.*, by glycosylation, acetylation, phosphorylation, etc. Any of numerous chemical modifications may be carried out by known techniques, including specific chemical cleavage by cyanogen bromide, trypsin, oxidation, reduction; etc.

In addition, analogs and fragments of lats can be chemically synthesized. For example, a peptide corresponding to a portion of a lats protein which comprises the desired domain, or which mediates the desired activity *in vitro*, can be synthesized by use of a peptide synthesizer. Furthermore, if desired, nonclassical amino acids or chemical amino acid analogs can be introduced as a substitution or addition into the lats sequence.

In a specific embodiment, the lats derivative is a chimeric, or fusion, protein comprising a lats protein or fragment thereof (preferably consisting of at least a domain or motif of the lats protein, or at least 15, preferably 20, amino acids of the lats protein) joined at its amino- or carboxy-terminus via a peptide bond to an amino acid sequence of a different protein. In one embodiment, such a chimeric protein is produced by recombinant expression of a nucleic acid encoding the protein (comprising a lats-coding sequence joined in-frame to a coding sequence for a different protein). Such a chimeric product can be made by ligating the appropriate nucleic acid sequences encoding the desired amino acid sequences to each other by methods known in the art, in the proper coding frame, and expressing the chimeric product by methods commonly known in the art. Alternatively, such a chimeric product may be made by protein synthetic techniques, *e.g.*, by use of a peptide synthesizer. Chimeric genes comprising portions of lats fused to any heterologous protein-encoding sequences may be constructed. A specific embodiment relates to a chimeric protein comprising a fragment of lats of at least six amino acids. In another specific embodiment, the lats derivative is a chimeric protein comprising a fragment of lats corresponding to amino acids 15-585 of human lats.

In another specific embodiment, the lats derivative is a molecule comprising a region of homology with a lats protein. By way of example, in various embodiments, a first

protein region can be considered "homologous" to a second protein region when the amino acid sequence of the first region is at least 30%, 40%, 50%, 60%, 70%, 75%, 80%, 90%, or 95% identical, when compared to any sequence in the second region of an equal number of amino acids as the number contained in the first region with no insertions or deletions considered, or when compared to an aligned sequence of the second region that has been aligned by a computer homology program known in the art. For example, a molecule can comprise one or more regions homologous to a lats domain or a portion thereof.

Derivatives of Lats Containing One or More Domains of the Protein

In specific embodiments, the methods of the invention use lats derivatives and fragments that comprise, or alternatively consist of, one or more domains of a lats protein, including but not limited to a lats C-terminal domain 3 (LCD3), lats C-terminal domain 2 (LCD2), lats C-terminal domain 1 (LCD1), kinase domain, kinase subdomains, lats flanking domain (LFD) (amino-terminal to the kinase domain), lats split domain 1 (LSD1), lats split domain 2 (LSD2), SH3-binding domain, and opa repeat domain, functional (*e.g.*, binding) fragments of any of the foregoing, or any combination of the foregoing.

In human lats (h-lats), m-lats, m-lats2, and *Drosophila* lats, the LCD3 domain is the last three amino acids of the protein, which are Val-Tyr-Val in all four proteins. For human lats and *Drosophila* lats, the LCD2 domain is amino acid residues 1077-1086 and 1075-1084, respectively (all amino acid residues provided in this paragraph are for the human and *Drosophila* lats amino acid sequences depicted in Figures 12 and 14, respectively (SEQ ID NOS:2 and 8, respectively)); the LCD1 domain is amino acid residues 1032-1043 and 1035-1047, respectively; the kinase domain is amino acid residues 703-1014 and 711-1018, respectively; the LFD domain is amino acid residues 607-702 and 612-710 respectively; and the putative SH3-binding domain is amino acids 247-268 and 196-217, respectively. For the lats split domains in *Drosophila*, the LSD1 is amino acid residues 365-392 and the LSD2 is amino acids 536-544. In human lats, the LSD1 and LSD2 domains are split into anterior and posterior portions such that the LSD1 is amino acid residues 328-334 and 498-518 and LSD2 is amino acid residues 28-31 and 555-559.

In particular, the Therapeutics of the invention include molecules comprising specific fragments of lats that are those fragments in the respective lats protein most homologous to specific fragments of a human or mouse lats protein.

In a specific embodiment, a lats protein, derivative or fragment is provided that has a kinase domain and has a phosphorylated or dephosphorylated serine situated within 20 residues upstream of an Ala-Pro-Glu consensus in subdomain eight of its kinase domain, or in which the serine situated within 20 residues upstream of that consensus has been deleted or substituted by another amino acid. In various specific embodiments, the invention provides various phosphorylated and dephosphorylated forms of the lats protein, derivative, or fragment that are active or inactive kinase forms. Both phosphorylation and dephosphorylation of lats at different residues could potentially activate or inactivate lats.

Phosphorylation can be carried out by any methods known in the art, *e.g.*, by use of a kinase. Dephosphorylation can be carried out by use of any methods known in the art, *e.g.*, by use of a phosphatase.

Another specific embodiment relates to a derivative or fragment of a lats protein that is a dominant-active protein kinase. Such a derivative or analog comprises a lats kinase domain that has been mutated so as to be dominantly active (exhibit constitutively active kinase activity). It is known that acidic residues such as Glu and Asp sometimes mimic a phosphorylated residue, and changing the phosphorylatable Ser or Thr residue in subdomain eight into a Glu or Asp residue has been previously used to produce constitutively active kinases (Mansour et al., 1994, Science 265:966-970). Thus, changing a serine or threonine residue situated within 20 residues upstream of an Ala-Pro-Glu consensus in subdomain eight of a lats kinase domain into another residue (*e.g.*, Glu, Asp) may be used to make a dominant-active lats protein kinase. For example, changing Ser914 in *Drosophila* lats, or changing Ser909 in human lats, into a Glu residue could produce a dominant active lats kinase.

Another specific embodiment relates to a derivative or fragment of lats that is a dominant-negative protein kinase. Protein kinases can be mutated into dominant negative forms. Expression of a dominant negative protein kinase can suppress the activity of the wild-type form of the same kinase. Dominant negative forms of protein kinases are often obtained by expressing an inactive form of a kinase (Milarski and Saltiel, 1994, J. Biol. Chem. 269(33):21239-21243) or by expressing a noncatalytic domain of a kinase (Lu and Means, 1994, EMBO J. 12:2103-2113; Yarden et al., 1992, EMBO J. 11:2159-2166). Thus, a lats dominant-negative kinase can be obtained by mutating the kinase domain so as to be inactive (*e.g.*, by deletion and/or point mutation). For example, a lats derivative that is a dominant-negative kinase is a lats protein that lacks a kinase domain but comprises one or more of the other domains of the lats protein; *e.g.*, a lats protein derivative truncated at about the beginning of the kinase domain (*i.e.*, a lats fragment containing only sequences amino-terminal to the kinase domain). As another example, a lats derivative that is a dominant-negative kinase is a lats protein in which one of the residues conserved among serine/threonine kinases (see Hanks et al., 1988, Science 241:42-52) is mutated (deleted or substituted by a different residue).

In another specific embodiment, a molecule is provided that comprises one or more domains (or functional portion thereof) of a lats protein but that also lacks one or more domains (or functional portion thereof) of a lats protein. For example, such a protein may lack all or a portion of the kinase domain, but retain at least the SH3-binding domain of a lats protein. In another embodiment, a molecule is provided that comprises one or more domains (or functional portion thereof) of a lats protein, and that has one or more mutant (*e.g.*, due to deletion or point mutation(s)) domains of a lats protein (*e.g.*, such that the mutant domain has decreased function). The kinase domain may be mutant so as to have reduced, absent, or increased kinase activity.

Lats-cdc2 Complexes

The invention provides lats-cdc2 complexes. In a preferred embodiment, the lats-cdc2 complexes are complexes of human proteins. As used herein, fragment or derivative of a lats-cdc2 complex includes complexes where one or both members of the complex are fragments or derivatives of the wild-type lats or cdc2 protein. Such derivatives and fragments can be generated as described for lats derivatives and fragments above. Preferably, the lats-cdc2 complexes in which one or both members of the complex are a fragment or derivative of the wild type protein are functionally active lats-cdc2 complexes.

In particular aspects, the native proteins, derivatives or analogs of lats and/or cdc2 are of animals, *e.g.* mouse, rat, pig, cow, dog, monkey, human, fly, frog, or of plants. "Functionally active lats-cdc2 complex" as used herein refers to that material displaying one or more known functional attributes of a complex of full length lats with a full length cdc2, including but not exclusive to control of cell cycle progression, cell proliferation, etc.

In specific embodiments, the lats-cdc2 complex contains a lats protein that is phosphorylated, preferably that is phosphorylated on a serine or threonine residue within 20 amino acids upstream of an Ala-Pro-Glu consensus subdomain eight of a lats kinase domain, more preferably that is phosphorylated on a serine residue corresponding to serine 909 of human lats, as depicted in Figure 12 (SEQ ID NO:2). In another specific embodiment, the lats-cdc2 complex contains a lats derivative in which a serine or threonine residue within 20 residues upstream of an Ala-Pro-Glu consensus subdomain eight of a lats kinase domain is substituted with a glutamate or aspartate residue, preferably, in which a serine residue corresponding to serine 909 of human lats is replaced with a glutamate residue. In another specific embodiment, the therapeutic is a fragment of a lats protein comprising or consisting of the amino acid sequence corresponding to amino acids 15 to 585 of human lats, as depicted in Figure 12 (SEQ ID NO:2).

Methods are presented for screening lats-cdc2 complexes, as well as derivatives and fragments of the lats-cdc2 complexes for the ability to alter lats and/or cdc2 activity, *e.g.*, to alter cell proliferation. For example, such derivatives or fragments which have the desired immunogenicity or antigenicity can be used in immunoassays, for immunization, for inhibition of lats-cdc2 complex activity, etc. Derivatives or fragments that retain, or alternatively lack or inhibit, a property of interest (*e.g.*, participation in a lats-cdc2 complex) can be used as inducers, or inhibitors, respectively, of such a property and its physiological correlates. A specific embodiment relates to a lats-cdc2 complex of a fragment of lats and/or a fragment of cdc2 that can be bound by an anti-lats and/or anti-cdc2 antibody or antibody specific for a lats-cdc2 complex when such a fragment is included within a lats-cdc2 complex.

The lats-cdc2 complexes can be obtained by any method known in the art. The cdc2 nucleotide and amino acid sequence is available from GenBank, accession no. Y00272 (see also, Lee and Nurse, 1987, Nature 327:31-35). The lats-cdc2 complexes can be obtained, for example, by expressing an entire lats coding sequence and a cdc2 coding sequence in the

same cell, either under the control of the same promoter or two separate promoters. In yet another embodiment, a derivative, fragment or homolog of lats and/or a derivative, fragment or homolog of cdc2 are recombinantly expressed. Preferably, the derivative, fragment or homolog of lats and/or the cdc2 protein form a complex with a binding partner identified by a binding assay, such as co-immunoprecipitation with an anti-lats or anti-cdc2 antibody, or interaction in a yeast two-hybrid assay (Fields and Song, 1989, Nature 340:245-246; and Finley and Brent, in DNA Cloning 2, Rickwood and Hames, eds (Oxford University Press, Oxford, 1995)).

In a specific embodiment, fusion or chimeric proteins are provided that contain the domains of a lats protein, or, in a specific embodiment, the amino acid sequence corresponding to amino acids 15 to 585 of human lats, and a cdc2 protein that directly form a lats-cdc2 complex and, optionally, a heterofunctional reagent, such as a peptide linker, linking the two domains, where such a heterofunctional reagent, such as a reagent or linker promotes the interaction of the lats and cdc2 binding domains. These fusion proteins may be particularly useful where the stability of the interaction is desirable (due to the formation of the complex as an intramolecular reaction), for example in production of antibodies specific to the lats-cdc2 complex.

Generation of Antibodies to Lats Proteins and Lats-cdc2 Complexes and Derivatives

LATS proteins, including functional derivatives and fragments thereof (*e.g.* a LATS protein encoded by a sequence of any one of SEQ ID NOs:2, 4, 6, or 8, or a subsequence thereof) may be used as an immunogen to generate monoclonal or polyclonal antibodies and antibody fragments or derivatives (*e.g.*, chimeric, single chain, Fab fragments, etc.). For example, antibodies to a particular domain of a lats protein may be desired. In a specific embodiment, fragments of a lats protein identified as hydrophilic are used as immunogens for antibody production using art-known methods. Various known methods for antibody production can be used including cell culture of hybridomas; production of monoclonal antibodies in germ-free animals (PCT/US90/02545); the use of human hybridomas (Cole *et al.*, Proc. Natl. Acad. Sci. U.S.A. (1983) 80:2026-2030; Cole *et al.*, in Monoclonal Antibodies and Cancer Therapy (1985) Alan R. Liss, pp. 77-96), and production of humanized antibodies (Jones *et al.*, Nature (1986) 321:522-525; US Pat. No. 5,530,101).

Diagnostic, Prognostic, and Screening Uses of Lats-cdc2 Complexes

Lats-cdc2 complexes may be markers of specific disease states involving disruption of physiological processes, such as cell cycle progression and cell proliferation, and pathological processes, such as hyperproliferative disorders, including tumorigenesis and tumor progression, and hypoproliferative disorders, and thus have diagnostic utility.

Detecting levels of lats-cdc2 complexes, or individual lats and cdc2 proteins or the mRNA encoding lats and cdc2 may be used in diagnosis or prognosis, to follow the course of disease states, or to follow therapeutic response, etc.

Lats-cdc2 complexes, lats and cdc2 proteins, and derivatives, and sub-sequences thereof, *lats* and/or *cdc2* nucleic acids (and sequences complementary thereto), and anti-lats-cdc2 complex antibodies and combinations of antibodies directed against lats and cdc2 have uses in diagnostics. Such molecules can be used in assays, such as immunoassays, to detect, prognose, diagnose, or monitor various conditions, diseases, and disorders characterized by aberrant levels of lats-cdc2 complexes or monitor the treatment thereof.

In particular, such an immunoassay is carried out by a method comprising contacting a sample derived from a patient with an anti-lats-cdc2 complex antibody under conditions such that immunospecific binding can occur, and detecting or measuring the amount of any immunospecific binding by the antibody. In a specific aspect, such binding of antibody, in tissue sections, can be used to detect aberrant lats-cdc2 complex localization or aberrant (*e.g.*, high, low or absent) levels of lats-cdc2 complex. In a specific embodiment, an antibody to a lats-cdc2 complex can be used to assay in a patient tissue or serum sample for the presence of a lats-cdc2 complex where an aberrant level of lats-cdc2 complex is an indication of a diseased condition. By "aberrant levels" is meant an increased or decreased level relative to that present, or a standard level representing that present, in an analogous sample from a portion of the body or from a subject not having the disorder.

The immunoassays which can be used include competitive and non-competitive assay systems using techniques such as Western blots, radioimmunoassays, ELISA (enzyme linked immunosorbent assay), "sandwich" immunoassays, immunoprecipitation assays, precipitin reactions, gel diffusion precipitin reactions, etc.

Nucleic acids encoding lats and cdc2 proteins and related nucleotide sequences and sub-sequences, including complementary sequences, can also be used in hybridization assays. The *lats* and *cdc2* nucleotide sequences, or sub-sequences thereof comprising about at least 8 nucleotides, can be used as hybridization probes. Hybridization assays can be used to detect, prognose, diagnose, or monitor conditions, disorders, or disease states associated with aberrant levels of the mRNAs encoding the components of a lats-cdc2 complex. In particular, such a hybridization assay is carried out by a method comprising contacting a sample containing nucleic acid with a nucleic acid probe capable of hybridizing to *lats* and *cdc2* DNAs or RNAs, under conditions such that hybridization can occur, and detecting or measuring any resulting hybridization.

By way of example, levels of lats-cdc2 complexes and lats and cdc2 proteins can be detected by immunoassay, levels of lats and cdc2 mRNA can be detected by hybridization assays (*e.g.*, Northern blots, dot blots), binding of lats to cdc2 can be done by binding assays commonly known in the art, translocations and point mutations in *lats* and/or *cdc2* can be detected by Southern blotting, RFLP analysis, PCR using primers that preferably

generate a fragment spanning at least most of the *lats* and/or *cdc2* gene, sequencing of the *lats* and/or *cdc2* genomic DNA or cDNA obtained from the patient, etc.

Also embodied are methods to detect a lats-cdc2 complex in cell culture models that express a lats-cdc2 complex, or derivatives thereof, for the purpose of characterizing or preparing the lats-cdc2 complex for harvest. This embodiment includes cell sorting of prokaryotes such as but not restricted to, bacteria (Davey and Kell, 1996, Microbiol. Rev. 60:641-696), primary cultures and tissue specimens from eukaryotes, including mammalian species such as human (Steele et al., 1996, Clin. Obstet. Gynecol 39:801-813), and continuous cell cultures (Orfao and Ruiz-Arguelles, 1996, Clin. Biochem. 29:5-9).

Kits for diagnostic use are also provided, that comprise in one or more containers an anti-lats-cdc2 complex antibody and, optionally, a labeled binding partner to the antibody. Alternatively, the anti-lats-cdc2 complex antibody can be labeled (with a detectable marker, *e.g.*, a chemiluminescent, enzymatic, fluorescent, or radioactive moiety). A kit is also provided that comprises in one or more containers a nucleic acid probe or probes capable of hybridizing to *lats* and *cdc2* mRNAs. In a specific embodiment, a kit can comprise in one or more containers a pair of primers (*e.g.*, each in the size range of 6-30 nucleotides) that are capable of priming amplification [*e.g.*, by polymerase chain reaction (see *e.g.*, Innis et al., 1990, PCR Protocols, Academic Press, Inc., San Diego, CA), ligase chain reaction (see EP 320,308) use of ß-replicase, cyclic probe reaction, or other methods known in the art], under appropriate reaction conditions of at least a portion of a *lats* nucleic acid and a *cdc2* nucleic acid. A kit can optionally further comprise in a container a predetermined amount of a purified lats-cdc2 complex, lats and cdc2 proteins or nucleic acids thereof, *e.g.*, for use as a standard or control.

Demonstration of Therapeutic Utility

The Therapeutics of the invention are preferably tested *in vitro*, and then *in vivo*, for the desired therapeutic or prophylactic activity, prior to use in humans. For example, *in vitro* assays which can be used to determine whether administration of a specific Therapeutic is indicated, include *in vitro* cell culture assays in which a patient tissue sample is grown in culture, and exposed to or otherwise administered a Therapeutic, and the effect of such Therapeutic upon the tissue sample is observed. A lower level of proliferation or survival of the contacted cells indicates that the Therapeutic is effective to treat the condition in the patient. Alternatively, instead of culturing cells from a patient, Therapeutics may be screened using cells of a tumor or malignant cell line. Many assays standard in the art can be used to assess such survival and/or growth; for example, cell proliferation can be assayed by measuring ^{3}H-thymidine incorporation, by direct cell count, by detecting changes in transcriptional activity of known genes such as proto-oncogenes (*e.g.*, fos, myc) or cell cycle markers; cell viability can be assessed by trypan blue staining, differentiation can be assessed visually based on changes in morphology, etc.

In another embodiment of the invention, a Therapeutic of the invention is screened for activity to modulate (*e.g.*, promote, inhibit or antagonize) cdc2 levels and/or activity. The levels of cdc2 protein and mRNA and cdc2 activity can be determined by any method well known in the art. For example, cdc2 protein can be quantitated by known immunodiagnostic methods such as western blotting immunoprecipitation using any antibody against cdc2 (for example, anti-cdc2 antibodies are commercially available from Santa Cruz Inc.) Cdc2 mRNA can be quantitated by methods that are well known and routine in the art, for example by northern analysis, RNase protection, the polymerase chain reaction in connection with the reverse transcription, etc. Cdc2 activity can also be assayed by any method known in the art, for example, by the histone-H1 kinase assay.

Compounds for use in therapy can be tested in suitable animal model systems prior to testing in humans, including but not limited to in rats, mice, chicken, cows, monkeys, rabbits, etc.

In a preferred embodiment, *lats* knock-out mice, *e.g.*, as described in the Examples, are used to test therapeutics of the invention for activity to treat or prevent cancers, or to modulate cdc2 activity.

Lats Knock-out Animals

The invention provides recombinant non-human animals in which one or more *lats* genes have been inactivated, *e.g.*, "knock-out animals". The recombinant non-human animal can be any animal, *e.g.*, mouse, rats, rodents, hamster, sheep, pig, cow, *Drosophila*, *C. elegans*, insects, worms, primates, dogs, etc., and is preferably a mouse. Such an animal can be generated by any method known in the art for disrupting a gene on the chromosome of an animal. *Lats* knock-out animals do not include animals in which one or more *lats* genes have been inactivated by naturally occurring mutations. In a preferred aspect, a *lats* knock-out animal can be produced by promoting homologous recombination between a *lats* gene in its chromosome and an exogenous *lats* gene that has been rendered biologically inactive (preferably by insertion of a heterologous sequence, *e.g.*, an antibiotic resistance gene). Homologous recombination methods for disrupting genes in the mouse genome are described, for example, in Capecchi (1989, Science 244:1288-1292) and Mansour et al. (1988, Nature 336:348-352). A *lats* knock-out mouse may be produced by the method described in the Examples section.

Briefly, all or a portion of a *lats* genomic clone is isolated from genomic DNA from the same species as the knock-out animal. The *lats* genomic clone can be isolated by any method known in the art for isolation of genomic clones (*e.g.*, by probing a genomic library with a probe derived from a lats sequence, such as those sequences provided in Figures 12-15, *i.e.*, SEQ ID NOS:1, 3, 5, or 7). Once the genomic clone is isolated, all or a portion of the clone is introduced into a recombinant vector. Preferably, the portion of the clone introduced into the vector that contains at least a portion of an exon of the *lats* gene, *i.e.*, contains a lats protein coding sequence. A sequence not homologous to the *lats* sequence,

preferably a positive selectable marker, such as a gene encoding an antibiotic resistance gene, is then introduced into the *lats* gene exon. The selectable marker is preferably operably linked to a promoter, more preferably a constitutive promoter. The non-homologous sequence is introduced anywhere in the lats coding sequence that will disrupt lats activity, *e.g.*, at a position where point mutations or other mutations have been demonstrated to inactivate lats protein function. For example, the non-homologous sequence can be inserted for the coding sequence for the portion of the lats protein containing all or a portion of the kinase domain (*e.g.*, the nucleotide sequence coding for at least 50, 100, 150, 200 or 250 amino acids of the kinase domain), the Lats C-terminal domain 1, the Lats C-terminal domain 2, and the Lats C-terminal domain 3, or, more preferably, for the sequence coding for the amino acids corresponding to 756 to 1130 of human lats (as depicted in Figure 12 (SEQ ID NO:2) and as indicated in the alignment of human and mouse lats in Figure 6A).

The positive selectable marker is preferably a neomycin resistance gene (*neo* gene) or a hygromycin resistance gene (hygro gene). The promoter may be any promoter known in the art; by way of example the promoter may be the phosphoglycerate kinase (PKG) promoter (Adra et al.,1987, Gene 60:65-74), the PolII promoter (Soriano et al., 1991. Cell 64:693-701), or the MC1 promoter, which is a synthetic promoter designed for expression in embryo-derived stem cells (Thomas & Capecchi, 1987, Cell 51:503-512). Use of a selectable marker, such as an antibiotic resistance gene, allows for the selection of cells that have incorporated the targeting vector (for example, the expression of the neo gene product confers resistance to G418, and expression of the hygro gene product confers resistance to hygromycin).

In a preferred embodiment, a negative selectable marker for a counterselection step for homologous, as opposed to non-homologous, recombination of the vector is inserted outside of the *lats* genomic clone insert, *e.g.*, as shown in Figure 6B. For example, such a negative selectable marker is the HSV thymidine kinase gene (HSV-tk), the expression of which makes cells sensitive to ganciclovir. The negative selectable marker is preferably under the control of a promoter such as the PGK promoter, the PolII promoter or the MC1 promoter.

When homologous recombination occurs, the portions of the vector that are homologous to the *lats* gene, as well as the non-homologous insert within the *lats* gene sequences, are incorporated into the *lats* gene in the chromosome, and the remainder of the vector is lost. Thus, since the negative selectable marker is outside the region of homology with the *lats* gene, cells in which homologous recombination has occurred (or their progeny), will not contain the negative selectable marker. For example, if the negative selectable marker is the HSV-tk gene, the cells in which homologous recombination has occurred will not express thymidine kinase and will survive exposure to ganciclovir. This procedure permits the selection of cells in which homologous recombination has occurred, as compared to non-homologous recombination in which it is likely that the negative

selectable marker is also incorporated into the genome along with the lats sequences and the positive selectable marker. Thus, cells in which non-homologous recombination has occurred would most likely express thymidine kinase and be sensitive to ganciclovir.

Once the targeting vector is prepared, it is linearized with a restriction enzyme for which there is a unique site in the targeting vector, and the linearized vector is introduced into embryo-derived stem (ES) cells (Gossler et al., 1986, Proc. Natl. Acad. Sci. USA 83:9065-9069) by any method known in the art, for example by electroporation. If the targeting vector includes a positive selectable marker and a negative, counterselectable marker, the ES cells in which homologous recombination has occurred can be selected by incubation in selective media. For example, if the selectable markers are the neo resistance gene and the HSV-tk gene, the cells are exposed to G418 (*e.g.*, approximately 300 μg/ml) and ganciclovir (*e.g.*, approximately 2 μM).

Any technique known in the art for genotyping, for example Southern blot analysis or the polymerase chain reaction, can be used to confirm that the disrupted lats sequences have homologously recombined into the *lats* gene in the genome of the ES cells. Because the restriction map of the *lats* genomic clone is known (see Figure 6b) and the sequence of the lats coding sequence is known (see Figure 13), the size of a particular restriction fragment or a PCR amplification product generated from DNA from both the disrupted and non-disrupted alleles can be determined. Thus, by assaying for a restriction fragment or PCR product, the size of which differs between the disrupted and non-disrupted *lats* gene, one can determine whether homologous recombination has occurred to disrupt the *lats* gene.

The ES cells with the disrupted lats locus can then be introduced into mouse blastocysts by microinjection and then the blastocysts can be implanted into the uteri of pseudopregnant mice using routine techniques. The mice that develop from the implanted blastocysts are chimeric for the disrupted allele. The chimeric male mice can be crossed to female mice, and this cross can be designed such that germline transmission of the allele is linked to transmission of a certain coat color. The germline transmission of the allele can be confirmed by Southern blotting or PCR analysis, as described above, of genomic DNA isolated from tail samples.

Isolating *Lats* Genes

Clones comprising lats nucleotide sequences, particularly *lats* genomic clones, can be isolated by any method known in the art. The nucleotide sequences encoding, and the corresponding amino acid sequences of, human lats, mouse lats, mouse lats2 and *Drosophila* lats are provided in Figures 12-15, respectively (SEQ ID NOS:1-8, respectively) and bacterial cells containing the plasmid pBS(KS)-h-lats, which contains the gene encoding human lats, were deposited on March 24, 1995 with the American Type Culture Collection, 10801 University Boulevard, Manassas, Virginia 20110-2201, and assigned Accession No. 69769. *Lats* nucleic acids, either *lats* genomic clones or *lats* specific probes to identify *lats* genomic clones, can be obtained by any method known in the art, *e.g.*, from

the deposited plasmid, by the polymerase chain reaction (PCR) using synthetic primers hybridizable to the 3' and 5' ends of a lats nucleotide sequence and/or by cloning from a cDNA or genomic library using an oligonucleotide probe specific for the gene sequence, such as a probe from the *lats* gene insert in plasmid pBS(KS)-h-lats. Genomic clones can be identified by probing a genomic DNA library under appropriate hybridization conditions, *e.g.*, high stringency conditions, low stringency conditions or moderate stringency conditions, depending on the relatedness of the probe to the genomic DNA being probed. For example, if the lats probe and the genomic DNA are from the same species, then high stringency hybridization conditions may be used; however, if the lats probe and the genomic DNA are from different species, then low stringency hybridization conditions may be used. High, low and moderate stringency conditions are all well known in the art.

Procedures for low stringency hybridization are as follows (see also Shilo and Weinberg, 1981, Proc. Natl. Acad. Sci. USA 78:6789-6792): Filters containing DNA are pretreated for 6 hours at 40°C in a solution containing 35% formamide, 5X SSC, 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 0.1% PVP, 0.1% Ficoll, 1% BSA, and 500 µg/ml denatured salmon sperm DNA. Hybridizations are carried out in the same solution with the following modifications: 0.02% PVP, 0.02% Ficoll, 0.2% BSA, 100 µg/ml salmon sperm DNA, 10% (wt/vol) dextran sulfate, and 5-20 X 106 cpm ^{32}P-labeled probe is used. Filters are incubated in hybridization mixture for 18-20 hours at 40°C, and then washed for 1.5 hours at 55°C in a solution containing 2X SSC, 25 mM Tris-HCl (pH 7.4), 5 mM EDTA, and 0.1% SDS. The wash solution is replaced with fresh solution and incubated an additional 1.5 hours at 60°C. Filters are blotted dry and exposed for autoradiography. If necessary, filters are washed for a third time at 65-68°C and reexposed to film.

Procedures for high stringency hybridizations are as follows: Prehybridization of filters containing DNA is carried out for 8 hours to overnight at 65°C in buffer composed of 6X SSC, 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.02% PVP, 0.02% Ficoll, 0.02% BSA, and 500 µg/ml denatured salmon sperm DNA. Filters are hybridized for 48 hours at 65°C in prehybridization mixture containing 100 µg/ml denatured salmon sperm DNA and 5-20 X 10^6 cpm of ^{32}P-labeled probe. Washing of filters is done at 37°C for 1 hour in a solution containing 2X SSC, 0.01% PVP, 0.01% Ficoll, and 0.01% BSA. This is followed by a wash in 0.1 X SSC at 50°C for 45 minutes before autoradiography.

Moderate stringency conditions for hybridization are as follows: Filters containing DNA are pretreated for 6 hours at 55°C in a solution containing 6X SSC, 5X Denhardt's solution, 0.5% SDS, and 100 µg/ml denatured salmon sperm DNA. Hybridizations are carried out in the same solution and 5-20 X 10 cmp ^{32}P-labeled probe is used. Filters are incubated in the hybridization mixture for 18-20 hours at 55°C, and then washed twice for 30 minutes at 60°C in a solution containing 1 X SSC and 0.1% SDS.

Any eukaryotic cell potentially can serve as the nucleic acid source for the molecular cloning of the *lats* gene. The nucleic acid sequences encoding lats can be isolated from vertebrate, mammalian, human, porcine, bovine, feline, avian, equine, canine, as well as

additional primate sources, insects, etc. The DNA may be obtained by standard procedures known in the art, preferably from cloned genomic DNA (*e.g.*, a DNA "library") from the desired cell (see, for example, Sambrook et al., 1989, Molecular Cloning, A Laboratory Manual, 2d Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York; Glover, D.M. (ed.), 1985, DNA Cloning: A Practical Approach, MRL Press, Ltd., Oxford, U.K. Vol. I, II.). The gene should be molecularly cloned into a suitable vector for propagation of the gene.

In preferred embodiments, the genomic clone used to generate a recombinant, non-human animal by homologous recombination contains at least a portion of the lats coding sequence of SEQ ID NO:3; alternatively, the genomic clone contains at least a portion of the lats coding sequence of SEQ ID NO:5.

Methods of Screening Therapeutics Using *Lats* Knock-out Mice

The invention provides methods for screening for compounds useful in the treatment or prevention of cancer or in the treatment and prevention of pituitary diseases and disorders by administration or application of the compound to be tested to a *lats* knock-out animal, preferably a *lats* knock-out mouse.

In a preferred embodiment, the invention provides a method for screening a potential therapeutic compound for activity in treating or preventing cancer. The potential therapeutic compound is administered to a recombinant non-human animal having at least one inactivated *lats* gene (*i.e.*, a *lats* knock-out animal, preferably a *lats* knock-out mouse), preferably two inactivated lats gene *lats* genes (*i.e.*, is homozygous for the inactivated *lats* allele). The size or progression of the cancer is then compared to that before the compound was added, or to a comparable recombinant animal without the administration of the compound, or to a normal, non-recombinant animal. A decrease in the size or progression of the cancer in the recombinant non-human animal after the administration of the compound as compared to the same animal prior to the administration or to another recombinant non-human animal not so administered or the standard size or progression of the cancer indicates that the compound has activity in treating or preventing cancer

The screening method of the invention can be used to screen for potential therapeutic compounds for the treatment or prevention of any cancer, preferably a cancer or neoplastic disease that is caused by the *lats* knock-out mutation. As described in the Examples section, *infra*, *lats* knock-out mice are susceptible to ovarian stromal tumors and soft tissue sarcomas that metastasize to vital organs. Accordingly, in preferred embodiments, the invention provides methods for screening compounds useful in treating or preventing ovarian tumors and soft tissue sarcomas. *Lats* knock-out mutations in other animals or in other *lats* homologs may make the resulting knock-out animal susceptible to other types of neoplastic disease. The invention also contemplates use of these other *lats* knock-out animals to screen compounds for efficacy in treating or preventing the types of neoplastic diseases found in these *lats* knock-out animals. Additionally, compounds

effective to treat or prevent ovarian tumors and/or soft tissue sarcomas in *lats* knock-out animals may also be effective to treat or prevent other types of cancers and neoplastic disease. Thus, *lats* knock-out animals may be used to screen for compounds that have activity to treat or prevent these other types of cancers and neoplastic disease.

The invention also provides methods of screening compounds for efficacy in treating or preventing skin cancer. As demonstrated in Examples section, *infra*, exposure to carcinogens induced, at a high frequency, skin tumors in the *lats* knock-out mice. Many methods are known in the art for inducing skin carcinogenesis in animals (for review see DiGiovanni, 1992, Pharmac. Ther. 54:63-128). Generally, mouse skin tumors can be elicited by application of a carcinogenic dose of tumor initiator, *e.g.*, 600 to 800 nmole of a pure polycyclic aromatic hydrocarbon such as 9,10-dimethyl-1,2-benzanthracene (DMBA). Other tumor initiators include, but are not limited to, arylamines, carbamates, haloalkylethers, haloaromatics, lactones, nitro-aromatics, nitrosamides and ureas. Alternatively, and preferably, mouse skin tumors can be induced by an initial application of a single sub-carcinogenic dose of a tumor initiator, *e.g.*, DMBA, and then repeated doses or exposures to a tumor promoter, such as phorbolesters (*e.g.*, TPA), teleocidins, polyacetates, okadaic acid, calyculin A, palytoxin, and thapsigargin. Ultraviolet B (UVB) radiation, skin abrasion and skin wounding are also strong tumor promoters.

In a preferred embodiment, such skin tumors are induced by a two-step process comprising a single treatment with DMBA, preferably 50 µl of a 0.5% DMBA solution in acetone, to the dorsal surface of the mouse 1 to 5 days after birth followed by repeated exposure to UVB irradiation, *e.g.*, exposures of approximately three times per week with an initial exposure of approximately 100 mJ/cm^2 per session, increasing the dosage by 10% per treatment (unless erythema or scaling occurs) to a maximum of 700 mJ/cm2, with an average of about 27 treatment sessions per mouse (Serrano et al., 1996, Cell 85:27-37).

Accordingly, in a preferred embodiment, the invention provides a method for screening a potential therapeutic compound for activity in treating or preventing skin cancer comprising administering the compound to a recombinant non-human animal in which one, preferably two, *lats* genes have been inactivated (i.e., a *lats* knock-out animal) and in which recombinant non-human animal tumors have been induced by exposure to at lease one carcinogen. The size or progression of the skin tumors are then compared before and after the administration of the compound. A reduction in the size or progression of the skin tumors in the recombinant non-human animal administered the compound as compared to same animal prior to administration of the compound or to the animal not so administered or to the standard size or progression of the skin tumors, indicates that the compound has activity in treating or preventing skin cancer. In another embodiment, the compound to be screened is administered by recombinantly expressing the compound in the recombinant non-human animal inactivated for the *lats* gene.

The administration of the compound to be tested can be carried out by any method known in the art, *e.g.*, orally, intravenously, intramuscularly, intraperitoneally,

subcutaneously, rectally, topically, etc. For the screening of compounds for efficacy in treating or preventing skin cancer, the compound is preferably applied topically.

After administration of the compound to be tested, the tumors, sarcomas, and other cancers can be evaluated by any diagnostic or histopathological method for detecting and evaluating tumors and cancers, for example, by visual inspection of the tumors (particularly for skin tumors), manual palpitation of tumors, biopsy or surgical removal of the tumor tissue and subsequent inspection, and sacrifice and dissection of the recombinant non-human animal. Morphological evaluation of tissue, either removed by biopsy or dissected from a sacrificed mouse, may be performed by fixing the tissue by any method known in the art, for example, in 10% neutral buffered formalin at 4°C, and subsequent dehydration, *e.g.*, in ethanol. The fixed and dehydrated tissue may be embedded in paraffin and then sectioned, for example into 4-5 mm sections by any method known in the art. Sections can be stained, for example, with a standard stain, such as hematoxylin and eosin, for microscopic inspection.

Another aspect of the invention provides methods for screening potential therapeutic compounds for efficacy in treating or preventing diseases or disorders associated with pituitary dysfunction. *Lats* knock-out mice display a number of consequences of pituitary dysfunction, as described in the Examples section, *infra.* The methods of the invention can be used to screen compounds for efficacy in treating or preventing such pituitary dysfunctions as pituitary hyperplasia, fertility defects, such as defective ovulation, lack of breast development, abnormal reproductive cycles in females, LH hypogonadotropic hypogonadism, reduced levels of pituitary hormones, specifically LH, GH and PRL, and reduced growth and metabolic abnormalities caused by reduced GH levels. Therapeutics that are effective to treat one or more of these conditions associated with pituitary dysfunction may also be effective to treat or prevent other conditions, diseases or disorders associated with pituitary dysfunction.

In a preferred embodiment, potential therapeutic compounds to be screened for activity in treating or preventing diseases and disorders associated with pituitary dysfunction are administered to a recombinant non-human animal in which one or more chromosomal copies of the *lats* gene have been inactivated (*i.e.*, a *lats* knock-out animal, preferably and *lats* knock-out mouse). Levels of an indicator of pituitary function or dysfunction are then compared in the recombinant non-human animal before and after the compound was administered. In one embodiment, the compound to be screened is administered by recombinantly expressing the compound in the recombinant non-human animal having an inactivated *lats* gene.

Indicators of pituitary function that may be assayed include fertility, ovulation, the female reproductive cycle (*e.g.*, the estrus cycle), breast tissue development, growth or size of the animal, including weight, skeletal size, *e.g.*, of the skull and/or longitudinal bones, and organ weight, and serum levels of LH, GH and PRL. These indicators may be measured by any means known in the art for evaluating these indicators. For example,

fertility may be evaluated by attempting to mate an animal and determining whether conception occurred, measuring sperm count in male animals or detecting ovulation in female animals. The reproductive organ tissue may also be examined histopathologically (*e.g.*, by fixing, sectioning and staining the tissue for inspection) for morphological defects, particularly in the testis, ovaries, and breast tissue. Whether the animal goes through an estrus cycle may be determined by observation of the animal. Hormone levels may be determined by any method known in the art, for example in serum samples by radio immunoassay using antibodies specific for the particular hormone. Lack of normal growth can be determined by measuring the animal *e.g.*, the weight, size of the skull and/or longitudinal bones, or organ weight, during maturation.

Candidate Therapeutics

Candidate therapeutics may come from any source of therapeutics known in the art. For example, these therapeutics can be proteins, nucleic acids (including anti-sense nucleic acids), antibodies, peptides, organic molecules, etc. In some instances, compounds may be screened first in *in vitro* assays to determine their potential as anti-cancer or anti-pituitary dysfunction therapeutics.

For example, chemical libraries may be screened for useful therapeutics. Exemplary libraries are commercially available from several sources (ArQule, Tripos/PanLabs, ChemDesign, Pharmacopoeia). Many diversity libraries suitable for use are known in the art and can be used to provide compounds to be tested according to the present invention. Alternatively, libraries can be constructed using standard methods. Chemical (synthetic) libraries (Houghten et al., 1991, Nature 354:84-86; Lam et al., 1991, Nature 354:82-84; Medynski, 1994, Bio/Technology 12:709-710; Gallop et al., 1994, J. Medicinal Chemistry 37(9):1233-1251), recombinant expression libraries, or polysome-based libraries are exemplary types of libraries that can be used. Other examples include combinatorial libraries (Ohlmeyer et al., 1993, Proc. Natl. Acad. Sci. USA 90:10922-10926; Erb et al., 1994, Proc. Natl. Acad. Sci. USA 91:11422-11426; Houghten et al., 1992, Biotechniques 13:412; Jayawickreme et al., 1994, Proc. Natl. Acad. Sci. USA 91:1614-1618; Salmon et al., 1993, Proc. Natl. Acad. Sci. USA 90:11708-11712), organic diversity (*e.g.*, nonpeptide) libraries (Bunin et al., 1994, Proc. Natl. Acad. Sci. USA 91:4708-4712) may be used. Libraries of non-peptides, *e.g.*, peptide derivatives (for example, that contain one or more non-naturally occurring amino acids) can also be used. One example of these are peptoid libraries (Simon et al., 1992, Proc. Natl. Acad. Sci. USA 89:9367-9371). Peptoids are polymers of non-natural amino acids that have naturally occurring side chains attached not to the alpha carbon but to the backbone amino nitrogen. Since peptoids are not easily degraded by human digestive enzymes, they are advantageously more easily adaptable to drug use.

Therapeutic/Prophylactic Administration and Compositions

The invention provides methods of treatment (and prophylaxis) by administration to a subject of an effective amount of a Therapeutic of the invention. In a preferred aspect, the Therapeutic is substantially purified. The subject is preferably an animal, including but not limited to animals such as cows, pigs, horses, chickens, cats, dogs, etc., and is preferably a mammal, and most preferably human. In a specific embodiment, a non-human mammal is the subject.

Various delivery systems are known and can be used to administer a Therapeutic of the invention, *e.g.*, encapsulation in liposomes (Langer, Science 249:1527-1533 (1990); Treat et al., in Liposomes in the Therapy of Infectious Disease and Cancer, Lopez-Berestein and Fidler (eds.), Liss, New York, pp. 353-365 (1989)), microparticles, microcapsules, recombinant cells capable of expressing the Therapeutic, receptor-mediated endocytosis (see, *e.g.*, Wu and Wu, 1987, J. Biol. Chem. 262:4429-4432), construction of a Therapeutic nucleic acid as part of a retroviral or other vector, etc. Methods of introduction include intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, intranasal, epidural, and oral routes. The compounds may be administered by any convenient route, for example by infusion or bolus injection, by absorption through epithelial or mucocutaneous linings (*e.g.*, oral mucosa, rectal and intestinal mucosa, etc.) and may be administered together with other biologically active agents. Administration can be systemic or local.

In a specific embodiment where the Therapeutic is a nucleic acid encoding a protein Therapeutic, the nucleic acid can be administered *in vivo* to promote expression of its encoded protein, by constructing it as part of an appropriate nucleic acid expression vector and administering it so that it becomes intracellular, *e.g.*, by use of a retroviral vector (see U.S. Patent No. 4,980,286), or by direct injection, or by use of microparticle bombardment (*e.g.*, a gene gun; Biolistic, Dupont), etc. Alternatively, a nucleic acid Therapeutic can be introduced intracellularly and incorporated within host cell DNA for expression, by homologous recombination.

The present invention also provides pharmaceutical compositions. Such compositions comprise a therapeutically effective amount of a Therapeutic, and a pharmaceutically acceptable carrier. In a specific embodiment, the term "pharmaceutically acceptable" means approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in animals, and more particularly in humans. The term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the Therapeutic is administered. The composition, if desired, can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents. These compositions can take the form of solutions, suspensions, emulsion, tablets, pills, capsules, powders, sustained-release formulations and the like. Examples of suitable pharmaceutical carriers are described in "Remington's Pharmaceutical Sciences" by E.W. Martin. Such compositions will contain a therapeutically effective amount of the Therapeutic, preferably in purified form, together with a suitable amount of carrier so as to

provide the form for proper administration to the patient. The formulation should suit the mode of administration.

In a preferred embodiment, the composition is formulated in accordance with routine procedures as a pharmaceutical composition adapted for intravenous administration to human beings. Typically, compositions for intravenous administration are solutions in sterile isotonic aqueous buffer. Where necessary, the composition may also include a solubilizing agent and a local anesthetic such as lignocaine to ease pain at the site of the injection.

The amount of the Therapeutic of the invention which will be effective in the treatment of a particular disorder or condition will depend on the nature of the disorder or condition, and can be determined by standard clinical techniques. In addition, *in vitro* assays may optionally be employed to help identify optimal dosage ranges. However, suitable dosage ranges for intravenous administration are generally about 20-500 micrograms of active compound per kilogram body weight. Suitable dosage ranges for intranasal administration are generally about 0.01 pg/kg body weight to 1 mg/kg body weight. Effective doses may be extrapolated from dose-response curves derived from *in vitro* or animal model test systems.

Suppositories generally contain active ingredient in the range of 0.5% to 10% by weight; oral formulations preferably contain 10% to 95% active ingredient.

The invention also provides a pharmaceutical pack or kit comprising one or more containers filled with one or more of the ingredients of the pharmaceutical compositions of the invention.

Screening for Lats Agonists and Antagonists

Lats nucleic acids, proteins, and derivatives may be used in screening assays to detect molecules that specifically bind to lats nucleic acids, proteins, or derivatives and thus have potential use as agonists or antagonists of lats, in particular, molecules that thus affect cell proliferation and/or cdc2 activity, or molecules that promote or inhibit formation of lats-cdc2 complexes. In a preferred embodiment, such assays are performed to screen for molecules with potential utility as lead compounds for drug development, particularly as anti-cancer drugs. The invention thus provides assays to detect molecules that specifically bind to lats nucleic acids, proteins, or derivatives or bind to or interfere with the formation of lats-cdc2 complexes. For example, recombinant cells expressing *lats* nucleic acids can be used to recombinantly produce lats proteins in these assays, to screen for molecules that bind to a lats protein, and recombinant cells expressing *lats* and *cdc2* nucleic acids can be used to recombinant produce both lats and cdc2 proteins in these assays, to screen for molecules that bind to or inhibit formation of a lats-cdc2 complex. Molecules (*e.g.*, putative binding partners of lats) are contacted with the lats protein (or fragment thereof) under conditions conducive to binding, and then molecules that specifically bind to the lats protein or bind to or interfere with the formation of lats-cdc2 complexes are identified.

Similar methods can be used to screen for molecules that bind to lats derivatives or nucleic acids. Methods that can be used to carry out the foregoing are commonly known in the art.

By way of example, diversity libraries, such as random or combinatorial peptide or nonpeptide libraries can be screened for molecules that specifically bind to lats. Many libraries are known in the art that can be used, *e.g.*, chemically synthesized libraries, recombinant (*e.g.*, phage display libraries), and *in vitro* translation-based libraries.

Examples of chemically synthesized libraries are described in Fodor et al., 1991, Science 251:767-773; Houghten et al., 1991, Nature 354:84-86; Lam et al., 1991, Nature 354:82-84; Medynski, 1994, Bio/Technology 12:709-710; Gallop et al., 1994, J. Medicinal Chemistry 37(9):1233-1251; Ohlmeyer et al., 1993, Proc. Natl. Acad. Sci. USA 90:10922-10926; Erb et al., 1994, Proc. Natl. Acad. Sci. USA 91:11422-11426; Houghten et al., 1992, Biotechniques 13:412; Jayawickreme et al., 1994, Proc. Natl. Acad. Sci. USA 91:1614-1618; Salmon et al., 1993, Proc. Natl. Acad. Sci. USA 90:11708-11712; PCT Publication No. WO 93/20242; and Brenner and Lerner, 1992, Proc. Natl. Acad. Sci. USA 89:5381-5383.

Examples of phage display libraries are described in Scott and Smith, 1990, Science 249:386-390; Devlin et al., 1990, Science, 249:404-406; Christian, R.B., et al., 1992, J. Mol. Biol. 227:711-718; Lenstra, 1992, J. Immunol. Meth. 152:149-157; Kay et al., 1993, Gene 128:59-65; and PCT Publication No. WO 94/18318 dated August 18, 1994.

In vitro translation-based libraries include but are not limited to those described in PCT Publication No. WO 91/05058 dated April 18, 1991; and Mattheakis et al., 1994, Proc. Natl. Acad. Sci. USA 91:9022-9026.

By way of examples of nonpeptide libraries, a benzodiazepine library (see *e.g.*, Bunin et al., 1994, Proc. Natl. Acad. Sci. USA 91:4708-4712) can be adapted for use. Peptoid libraries (Simon et al., 1992, Proc. Natl. Acad. Sci. USA 89:9367-9371) can also be used. Another example of a library that can be used, in which the amide functionalities in peptides have been permethylated to generate a chemically transformed combinatorial library, is described by Ostresh et al. (1994, Proc. Natl. Acad. Sci. USA 91:11138-11142).

Screening the libraries can be accomplished by any of a variety of commonly known methods. See, *e.g.*, the following references, which disclose screening of peptide libraries: Parmley and Smith, 1989, Adv. Exp. Med. Biol. 251:215-218; Scott and Smith, 1990, Science 249:386-390; Fowlkes et al., 1992; BioTechniques 13:422-427; Oldenburg et al., 1992, Proc. Natl. Acad. Sci. USA 89:5393-5397; Yu et al., 1994, Cell 76:933-945; Staudt et al., 1988, Science 241:577-580; Bock et al., 1992, Nature 355:564-566; Tuerk et al., 1992, Proc. Natl. Acad. Sci. USA 89:6988-6992; Ellington et al., 1992, Nature 355:850-852; U.S. Patent No. 5,096,815, U.S. Patent No. 5,223,409, and U.S. Patent No. 5,198,346, all to Ladner et al.; Rebar and Pabo, 1993, Science 263:671-673; and PCT Publication No. WO 94/18318.

In a specific embodiment, screening can be carried out by contacting the library members with a lats protein (or nucleic acid or derivative) immobilized on a solid phase and

harvesting those library members that bind to the protein (or nucleic acid or derivative). Examples of such screening methods, termed "panning" techniques are described by way of example in Parmley and Smith, 1988, Gene 73:305-318; Fowlkes et al., 1992, BioTechniques 13:422-427; PCT Publication No. WO 94/18318; and in references cited hereinabove.

In another embodiment, the two-hybrid system for selecting interacting proteins in yeast (Fields and Song, 1989, Nature 340:245-246; Chien et al., 1991, Proc. Natl. Acad. Sci. USA 88:9578-9582) can be used to identify molecules that specifically bind to a lats protein or derivative or that interfere with the formation of lats-cdc2 complexes. Additionally, the two hybrid system or co-immunoprecipitation of lats and cdc2 can be used as assays to screen for compounds that promote or inhibit formation of lats-cdc2 complexes.

In a preferred embodiment, the invention provides a method of screening for a molecule that modulates (*i.e.*, inhibits, antagonizes or promotes) directly or indirectly, the formation of a complex of lats and cdc2 proteins comprising measuring the levels of said complex formed from lats and cdc2 proteins in the presence of said molecule (optionally, purified) under conditions conducive to formation of the complex; and comparing the levels of said complex with the levels of said complex that are formed in the absence of said molecule, lower or higher level of said complex in the presence of said molecule indicates that the molecule modulates formation of said complex.

EXAMPLES

EXAMPLE 1: Human Lats Modulates Cdc2/Cyclin A Activity

Using mammalian cell culture assays, we have found that lats is phosphorylated in a cell cycle-dependent manner and that it complexes with cdc2 in early mitosis. Lats associated cdc2 has no mitotic cyclin partner and no kinase activity for histone H1. Furthermore, we have found that *lats* mutant cells in *Drosophila* abnormally accumulate cyclin A. These biochemical observations indicate that lats is a negative regulator of cdc2/cyclin A, a finding supported by *in vivo* genetic data demonstrating that lats specifically interacts with cdc2 and cyclin A in *Drosophila*.

Materials and Methods

For yeast two-hybrid experiments, DNA encoding N-terminal h-lats (amino acid numbers 15 to 585 of the human lats protein sequence as depicted in Figure 12 (SEQ ID NO:2)), C-terminal h-lats (amino acid number 589 to 1130 of the human lats protein sequence as depicted in Figure 12 (SEQ ID NO:2)), and h-lats (amino acid numbers 15 to 1130 of the human lats protein sequence as depicted in Figure 12 (SEQ ID NO:2)) were cloned into pBTM116 (Bartel et al., Cellular Interactions in Development: A Practical Approach, ed. D. Hartley (Oxford University Press, Oxford, England (1993)). DNA

encoding human cdc2, CDK2, CDK4 and C-terminal h-lats were cloned in pACT (Durfee et al., 1993, Genes & Devel. 8:440-452). The constructs were transformed into yeast strain L40 (Vojtek et al., 1993, Cell 74:205-214). The transformants were tested for growth on SD his-ura-trp-leu medium (Bio101, California) and for ß-galactosidase activities (Vojtek et al., 1993, Cell 74:205-214).

For baculovirus experiments, full-length *h-lats* cDNA was cloned into the vector pBacPAK8 and baculovirus were produced according to the protocols provided by Clontech. IPLB-Sf21 cells were co-infected with equal amounts of h-lats and human cdc2-baculoviruses and were harvested 62 hours after infection for immunoprecipitation and immunoblot assay.

Fly Genetics

The full-length *h-lats* cDNA was cloned into the vector pCaSpeR-hs (Tummel and Pirrott, 1992, *Drosophila* Information Service 71:150). Multiple transformant lines were obtained and used in rescue experiments with $lats^{e532}$, $lats^{e26\text{-}1}$, $lats^{a1}$, and $lats^{X1}$ alleles. Expression of *hs-h-lats* was induced as described in Xu et al. (1995, Development 121:1053-1063) -- incubation at 37°C for one hour every day until eclosure. Since the induction of *h-lats* requires heat-shock treatments, X-ray irradiation was used to induce mitotic clones in *y w P[hs-h-lats]/y w*; $P[FRT]82B\ lats^{x\text{-}1}/P[y+)96E$ animals. The rest of the *lats* mutant mitotic clones were induced and labeled according to Xu and Rubin (1993, Development 117:1223-1237). The *Drosophila lats* and *h-lats* cDNAs were cloned into the pGMR vector to generate multiple transformant lines (Hay et al., 1994, Development 120:2121-2129). Besides the mutations mentioned above, $cdc2c^{E136E}$ (a gift of Helena Richardson), $cycA^{nco114}$, *Df(2R)59A-B* were used for *cdc2c*, *cyclin A* and *cyclin B*, respectively.

Tissue Culture

HeLa cells were synchronized at different cell cycle stages by various treatments as described by Knehr et al.(1995, Exp. Cell. Res. 217:546-553). Briefly, cells were arrested at G1 by thymidine and hydroxyurea treatment; at S phase by thymidine double block (incubation in the presence of 2 mM thymidine for 24 hours, followed by recovery in the absence of thymidine for 12 hours, followed by another incubation in 2 mM thymidine for 14 hours) plus a 4 hour incubation in medium without thymidine; and at G2 by thymidine double block plus an 8 hour incubation in medium without thymidine. To arrest cells in M phase, cells were treated with 0.1 µg/ml nocodazole (Sigma) for 12 hours and mitotic cells were shaken off of the flask and washed twice with cold DMEM without serum. These mitotic cells were then resuspended in fresh warm medium without nocodazole and incubated in suspension at 37°C. Cells were harvested at various time points after removal of nocodazole (herein "ARN") for further analysis. CHO cells were grown in a-MEM medium plus 7% FBS and IPLB-Sf21 cells were grown in sf-900 II SFM plus 10% FBS.

Antibodies and Immunochemistry

Anti-human lats rat monoclonal and rabbit polyclonal antibodies were raised against a GST-N-h-Lats (GST fused to the N-terminal portion of lats, *i.e.*, consisting of amino acids 15-585 of the human lats amino acid sequence as depicted in Figure 12 (SEQ ID NO:2) fusion protein. Anti-human cdc2 (#sc 054), anti-human cyclin B (#sc 245), anti-human cyclin A (#sc 239) monoclonal antibodies were purchased from Santa Cruz Inc. Rabbit polyclonal anti-*Drosophila* Cyclin A and B antibodies were gifts of David Glover. Monoclonal mouse anti-BrdU antibodies (#347580) were purchased from Becton Dickinson and monoclonal mouse ant-c-myc antibodies (#OP 10) were purchased from Oncogene Sciences. Propidium iodide (Sigma) was used as a DNA marker.

HeLa, CHO, or IPLB Sf21 cells were lysed in TG buffer (1% Triton, 10% glycerol) (Sun et al., 1996, Genes & Devel. 10:395-406) plus freshly added proteinase inhibitors (1 mM PMSF, 10 μg/ml leupeptin, 1 μg/ml pepstatin, and 2 μg/ml aprotinin). The lysates were centrifuged at 50 K, 4°C for 12 minutes. Supernatants were pre-cleaned by incubating with protein G-agarose. Immunoprecipitation and western blots were performed by the procedures described by Sun et al. (1996, Genes & Devel. 10:395-406). Western blots were visualized by enhanced chemiluminescence (Amersham). Whenever necessary the blots were stripped following the procedure described by Edgar et al. (1994, Genes & Devel. 8:440-452). Calf Intestinal Phosphatase (CIP) treatments were carried out as described in Sun et al. (1996, Genes & Devel. 10:395-406).

H1 Kinase Assay

HeLa cell lysates (50 minutes ARN) were precleaned by incubation in protein G-agarose. Immunoprecipitates were washed three times with TG buffer and twice with 1X kinase buffer (50 mM Tris-HCl 7.5, 10 mM MgCl2, 5 mM EGTA, 2 mM DTT) without DDT. The kinase assay was carried out on ice for 10 minutes in 35 μl of 1X kinase buffer containing 15 μCi of γ-^{32}P ATP, 1.6 μg of histone H1, and 1.5 μM ATP. The kinase activities were measured by quantifying the intensities of histone-H1 phosphorylation using a PhosphorImager (Molecular Dynamics). The amounts of cdc2 in the immunoprecipitates were determined by anti-cdc2 immunoblotting and densitometer scanning (Molecular Dynamics). The kinase assay experiments were repeated three times.

Results

Human Lats Can Functionally Replace its Fly Counterpart

Sequence conservation suggested that human lats could be a functional homolog of *Drosophila* lats. To test this, the human *lats* cDNA was introduced into the *Drosophila* genome under the control of the heat shock-inducible promoter (*hs-h-lats*) (Lis et al., 1983, Cell 35:403-410) and expressed the transgene was expressed under the conditions previously established for rescue using the fly *lats* gene (Xu et al., 1995, Development 121:1053-1064; and PCT Publication WO 96/30402, published October 3, 1996). In

mosaic flies, clones of cells mutant for *lats* undergo extensive overproliferation and develop into large tumors in various tissues (Figure 1A and Xu et al., 1995, Development 121:1053-1064). Expression of human lats completely suppressed tumor formation in *lats* mosaic flies (Figures 1B-D). Instead, the *lats* mutant cells (genetically marked as *yellow-* cells) in these human lats-expressing mosaic animals developed into normal adult structures (Figures 1C and D). The ability of the human gene to support normal fly development was further examined. Expression of the human *lats* transgene in homozygous *lats* mutant *Drosophila* rescued all developmental defects including embryonic lethality found in homozygous *lats* mutants. Furthermore, the extent of phenotypic rescue correlated with the level of human lats expression. Complete phenotypic rescue required daily induction of human lats, and leaky expression of lats controlled by the heat shock promoter at 25°C resulted in partial suppression of the *lats* mutant overproliferation phenotype (Figures 1E and F). These data demonstrate that human lats is an authentic homolog of the *Drosophila* lats tumor suppressor.

Lats is phosphorylated in a cell cycle-dependent manner

To further explore the function of lats, the biochemical properties of the human lats protein were examined. Lats immunoprecipitated from HeLa cells had two major migrating forms (Figure 2A, lane 6). The slow-migrating form of lats was converted into the fast-migrating form after the proteins were incubated with calf intestine alkaline phosphatase (CIP) (Figure 2A). Addition of a phosphatase specific inhibitor, ß-glycerophosphate, to the phosphatase reaction blocked this conversion (Figure 2A, lanes 5 and 10). These results indicate that the slow-migrating form is phosphorylated lats, while the fast-migrating form is dephosphorylated lats.

Lats immunoprecipitated from cells at different mitotic stages displayed varying amounts of the two forms (compare lanes 1 and 6 of Figure 2A), suggesting that the phosphorylation state of lats may oscillate with the cell-cycle. To verify this possibility, lats proteins were immunoprecipitated from extracts of HeLa cells at G0, G1, S, and G2 phases, and different time points during mitosis (minutes after removal of nocodazole (ARN) block) (Knehr, et al., 1995, Exp. Cell Res. 217:546-553). DAPI staining was used to verify the cell cycle progression. All lats protein was phosphorylated at late prophase (0 minutes ARN; Figure 2B), and remained phosphorylated through metaphase (50 minutes ARN; Figures 2B and C). Dephosphorylated lats could be detected when cells in the culture begin to enter anaphase (75 minutes ARN; Figures 2B and C), and by the start of telophase (100 minutes ARN) most of the lats protein was dephosphorylated (Figures 2B and C). In late mitosis, G1, S, G2 or G0 phase, lats molecules were in the dephosphorylated form (Figure 2B). These observations strongly suggest that the lats protein undergoes two major phosphorylation changes during the cell cycle. At the G2/M boundary or in early prophase, lats is phosphorylated, and lats becomes dephosphorylated at the metaphase/anaphase boundary or in early anaphase.

Lats complexes with cdc2 during mitosis

Lats mutant cells in *Drosophila* mosaic for the *lats* mutation do differentiate, indicating that mutations in *lats* do not block cellular differentiation in general (Figures 1G and H). The *lats* mutant overproliferation phenotype and cell cycle-dependent phosphorylation of lats suggest that the protein could be directly involved in the regulation of the cell cycle.

Immunoprecipitation experiments were carried out to examine whether lats proteins complex with known cell cycle regulators. Interestingly, cdc2 was found to co-immunoprecipitate with lats in mitotic cells (Figures 3A and B). The co-precipitation of lats and cdc2 was confirmed in both murine and human cells using several polyclonal and monoclonal anti-human lats antibodies (as described above in the Materials and Methods section, *supra*). Although similar amounts of lats were immunoprecipitated from HeLa cells during different stages of mitosis (Figure 3B, upper panel), the amount of co-precipitated cdc2 varied (Figure 3B, lower panel). Co-precipitated cdc2 was most abundant at early mitosis (0 and 50 minutes ARN; Figure 3B; also see Figures 2B and C for cell cycle progression). The amount of co-precipitated cdc2 then progressively decreased as the cell cycle progressed (Figure 3B--lanes 100', 150', and 200'). No cdc2 co-immunoprecipitation could be detected in quiescent cells in G0 (Figure 3A and Figure 2B). The difference in the amount of co-precipitated cdc2 cannot be attributed to changes of cdc2 levels during the cell cycle, since it has previously been shown that the cdc2 protein is maintained at a nearly constant level in cycling cells (Dalton, 1992, EMBO J. 11:1797-1804; McGowan et al., 1990, Mol. Cell. Biol. 10:3847-3851). Indeed, equal amounts of cdc2 protein were precipitated from the 50 minutes and 200 minutes ARN extracts when anti-cdc2 antibodies were used.

The interaction of lats and cdc2 proteins was studied by expressing human lats and cdc2 proteins in the baculovirus expression system. The baculovirus-expressed cdc2 and lats proteins could be co-immunoprecipitated using either anti-human lats or anti-cdc2 antibodies (Figure 3C). This result suggests that the *in vivo* lats/cdc2 complex may result from direct binding of the two proteins.

The interaction between lats and cdc2 was also examined using the yeast two-hybrid assay (Fields and Song, 1989, Nature 340:245-245; Finley and Brent, in DNA Cloning 2, Rickwood and Hames, eds (Oxford University Press (Oxford, 1995)). Consistent with the co-immunoprecipitation results, full length lats and the N-terminal region of lats interacted with cdc2 in the assay (Figure 3E). Since the C-terminal kinase domain of lats did not interact with cdc2, these results indicate that lats associates with cdc2 through its N-terminal domain. Furthermore, neither full length lats nor the N-terminal region of lats showed any interaction with two G1 cell cycle kinases, CDK2 and CDK4 (Figure 3E), indicating that the association between lats and cdc2 is specific.

The lats/cdc2 complex is inactive for H1 kinase activity

We examined whether the lats/cdc2 complex has any kinase activity on the substrate histone H1. Lats/cdc2 and cdc2/cyclin B complexes were immunoprecipitated separately from 50 minute ARN HeLa cell extracts using either anti-human lats or anti-cyclin B monoclonal antibodies and assayed for histone H1 kinase activities. In contrast to the cdc2/cyclin B complex, the lats/cdc2 complex showed no detectable kinase activity for histone H1 (Figure 3D). Densitometer readings indicated that the H1 kinase activity of the lats/cdc2 complex does not differ from the background control and is at least 25 fold lower than the kinase activity of the cdc2/cyclin B complex. These results indicate that cdc2 molecules associated with lats are inactive or have dramatically reduced mitotic kinase activity.

The lack of H1 kinase activity in the lats-associated cdc2 could be due to the inhibition of the kinase activity of the cdc2/cyclin complex by lats. Alternatively, the lats/cdc2 complex may lack cyclin A and B which are the indispensable subunits for cdc2 kinase activity (Draetta et al., 1989, Cell 56:829-838; Solomon et al., 1990, Cell 63:1013-1024). Neither cyclin A nor cyclin B proteins could be detected in the lats/cdc2 immunocomplex when probed with anti-cyclin A and B antibodies (Figure 3B), indicating that lats modulates cdc2 activity in a way different from that of the known cyclin dependent kinase inhibitors (CDIs) (Sherr, 1996, Science 274:1672-1677; Harper, 1997, Cancer Surveys 29:91-107).

Lats genetically interacts with cdc2 and cyclin A during *Drosophila* development

In *Drosophila*, cdc2 also complexes with cyclin A or B (Knoblich et al., 1994, Cell 77:107-120). We examined the potential genetic interactions between lats, cdc2, cyclin A and cyclin B in *Drosophila*. Animals heterozygous for the strong *cdc2* allele, $cdc2^{B47}$, or homozygous for the temperature sensitive $cdc2^{ts}$ mutation at permissive temperature are viable and morphologically normal (Clegg et al., 1993, Genome 36:676-685). The $lats^{P8}$ mutation causes late pupal lethality in homozygous mutants (Figure 4A), and reducing cdc2 activity in $lats^{P8}$ homozygotes by introducing one copy of a *cdc2* mutant allele ($cdc2^{B47}$ or $cdc2^{ts}$/+; $lats^{P8}/lats^{P8}$) was sufficient to rescue the lats-associated lethality (Figure 4B). Furthermore, the overproliferation phenotype of $lats^{P8}$ adult appendages were also suppressed. Rescued animals had near-wild type eyes in comparison to the overproliferated, large, rough eyes of the $lats^{P8}$ mutants (Figures 4C and D). Reducing cdc2 activity also suppressed the giant larvae/pupae and disc overproliferation phenotypes of the $lats^{E26-1}$ animals (Figures 4E and F). The *Drosophila* CDK2 homolog, cdc2c complexes with Cyclin E (Lehner and O'Farrell, 1990, Cell 61:535-547; Knoblich et al., 1994, Cell 77:107-120). Consistent with the result of the yeast two-hybrid assay for human lats and CDK2 (Figure 3C), inactivation of one copy of the *cdc2* gene did not modify the phenotypes of the $lats^{P8}$ mutant animals. We further examined the potential genetic

interaction between *lats*, *cyclin A* and *cyclin B* in *Drosophila* as described above. Interestingly, while cyclin B did not interact with lats, cyclin A behaved similarly to cdc2. Inactivation of one copy of the cyclin A gene resulted in almost identical phenotypic suppression of the $lats^{P8}$ and $lats^{E26-1}$ mutants as did the *cdc2* mutants (data not shown). Thus, the specific genetic interactions between lats, cdc2, and cyclin A confirms the biochemical data indicating that lats regulates cell proliferation by negatively modulating cdc2/cyclin A activity.

While cdc2 protein remains at a constant level during the cell cycle (Dalton, 1992, EMBO J. 11:1797-1804; McGowan et al., 1990, Mol. Cell. Biol. 10:3847-3851), cyclin A and B are degraded when the cdc2/cyclin complexes are inactivated (Draetta et al., 1989, Cell 56:829-828; Murray et al., 1989, Nature 339:280-286; King et al., 1994, Cell 79:563-571).

Accordingly, whether lats inactivation leads to accumulation of cyclin A and B was also determined. In the *Drosophila* eye imaginal disc, cyclin A and B are detected in cells anterior to the morphogenetic furrow (MF) as well as in a stripe of cells posterior to the MF which are undergoing the last round of cell division (the second mitotic wave) (Thomas et al., 1994, Cell 77:1003-1014). In clones of lats cells located anterior to the MF, anti-cyclin A or B antibody staining did not detect any obvious changes in levels of the two proteins (Figures 5A-N; Whitfield et al., 1990, EMBO J. 9:2563-2572), which might be due to the fact that cells in this region already accumulate high levels of both cyclin A and B. Cells in the MF are synchronized in G1 and so are the cells posterior to the MF which are differentiating into neurons. Both populations of the G1 cells have no detectable cyclin A or B (Thomas et al., 1994, Cell 77:1003-1014; Figures 5B and H). However, in clones of $lats^{+}$ cells in the MF and in the region posterior to the MF, a high level of cyclin A was detected (Figures 5A-E). Interestingly, in $lats^{-/-}$ cells, cyclin A was still degraded in cells at late mitosis (Figure 5F), indicating that lats affects limited aspects of the cell cycle. Finally, consistent with the genetic interaction results, $lats^{-/-}$ clones did not cause obvious changes in the staining pattern of cyclin B (Figures 5G and H). These results provide direct evidence indicating that inactivation of *lats* causes overproliferation by deregulating cdc2/cyclin A activity.

The role of lats in cell cycle regulation was examined by overexpressing lats in the developing eye imaginal disc. When *Drosophila* and human *lats* cDNAs were expressed in cells in and posterior to the MF under the direction of the GMR promoter (Hay et al., 1994, Development 120:2121-2129), they exhibited similar phenotypes. Adult eyes from these animals were smaller than wild type and had irregular architecture with missing bristles, a phenotype reminiscent to that of overexpressing p21 under the same promoter (*GMR-p21*; de Nooij and Hariharan, 1995, Science 270:983-985) (Figures 5I and J). Sections of GMR-lats adult retinas also revealed a phenotype identical to that observed in retinas from flies transformed with *GMR-p21*. While almost all of the ommatidia contained the full complement of photoreceptor cells, many pigment cells were missing (Figure 5K). This

phenotype suggested that, like overexpression of p21, overexpression of lats blocked the last round of cell division in the developing eye disc. Further examination of the GMR-lats eye discs revealed that in the region of second mitotic wave there was an accumulation of cells with intense propidium-iodide staining, indicating that the cells were tetraploid, which was followed immediately posteriorly by apoptotic cells with fragmented nuclei (Figure 5L). In contrast to the overexpression of p21 which blocked entry into S phase, overexpressing lats arrested cells at G2/M or M phase. Consistent with this conclusion, BrdU labeling experiments revealed that the S phase of the cells in the second mitotic wave did occur in GMR-lats eye discs (Figures 5M and N).

Discussion

The lats molecules are a novel family of conserved proteins

Expression of human lats under the same conditions used for rescue by the fly gene completely suppressed tumor formation in *lats* mosaic flies and rescued all developmental defects in *lats* homozygous mutant *Drosophila*. These experiments provide definitive evidence for functional conservation among the *lats* genes, indicating that human lats can perform all functions that are normally provided by the fly protein.

A model for lats function

Our biochemical and genetic data support the hypothesis that lats is a negative regulator of cdc2/cyclin A. Cdc2 co-immunoprecipitated with lats using either HeLa and CHO cell extracts or baculovirus-expressed proteins. Cdc2 also interacted with lats in yeast two-hybrid assays. Moreover, lats-associated cdc2 has no cyclin A or B subunit and no histone H1 kinase activity. In *Drosophila*, *lats* mutant cells abnormally accumulated cyclin A. Genetic data in *Drosophila* demonstrate that the overproliferation and lethality phenotypes of *lats* mutants can be suppressed by mutations in *cdc2* and *cyclin A* genes. The genetic interaction between lats, cdc2, and cyclin A is highly specific. While animals heterozygous for cdc2 or cyclin A did not display any defects, removal of one copy of either gene was sufficient to dominantly suppress the *lats* mutant phenotypes. Such a genetic interaction was not observed in hundreds of genes examined, including other positive cell cycle regulators such as *cdc2c*, *cyclin B*, and *dE2F*.

The lats kinase domain contains all 11 subdomains previously found in other protein kinases (Hanks et al., 1988, Science 241:42-45), suggesting that it is an active protein kinase. However, lats alone and lats/cdc2 complex do not appear to have any autophosphorylation activity or phosphorylation activity for cdc2 and histone H1. Yeast two-hybrid experiments showed that the N-terminal region of lats interacted with cdc2 much more strongly than did full-length lats (Figure 3E). This result indicates that the C-terminal kinase domain of lats has a negative effect on the binding between the lats N-terminal region and cdc2.

The association of lats with cdc2 is directly correlated with its state of phosphorylation (compare Figures 2B and 3B). During early mitosis, lats is phosphorylated and associates with cdc2. At G0, lats is dephosphorylated and fails to associate with cdc2. Furthermore, the transition of the lats phosphorylation state during mitosis correlates with a change in its ability to bind to cdc2. Phosphorylation is a common mechanism that regulates protein activities during the cell cycle (Hunter, 1995, Cell 80:225-236).

Cell-cell communication mechanism regulating cell proliferation

Many tumor suppressors probably evolved to play important regulatory roles during development. The study of the normal developmental functions of a tumor suppressor is essential to our understanding of the mechanisms of tumorigenesis. A growing body of evidence suggests that proliferating cells in a developing *Drosophila* imaginal disc communicate to maintain a constant disc size, and that lats plays an important role in this process. Using mutations such as *Minute* and *dE2F* in *Drosophila*, it has been shown that, from a young mosaic disc containing cells of different genotypes, the number of progeny cells from a given parental cell can vary dramatically in a mature disc, while the overall size of the mature disc is unaffected (Simpson, 1979, Devel. Biol. 69:182-193; Simpson and Morata, 1981, Devel. Biol. 85:299-308; Brook et al., 1996, EMBO J. 15:3676-3683). Imaginal discs can also undergo regeneration when a small region of a disc is surgically removed, a phenomenon similar to liver regeneration in mammals (French et al., 1976, Science 193:969-981; Meinhardt, 1994, Bioessays 16:627-632; Michalopoulos and DeFrances, 1997, Science 276:60-66). Consistent with the notion that proliferation is regulated by local cell interaction, it has been shown that DNA replication and mitosis in growing discs occur in small, non-clonal clusters of cells throughout the disc (Adler and MacQueen, 1981, Exp. Cell Res. 133:452-456; Milan et al., 1996, Proc. Natl. Acad. Sci. USA 93:11687-11692; Milan et al., 1996, Proc. Natl. Acad. Sci. USA 93:640-645). Furthermore, young discs transplanted into adult hosts grow until the disc reaches its normal size, indicating that such a size control mechanism is an intrinsic property of the cells in each disc (Bryant in The Genetics and Biology of *Drosophila*, Vol. 2c, Ashburner and Novitski, eds. (Academic Press, New York, 1978). Transplantation experiments in mice revealed a similar size control phenomenon with anlagen of some vertebrate organs (Leitina et al., 1971, Transplantation 11:499-502).

Lats mutations dramatically disrupt the size and shape of discs in *Drosophila*. Clones of *lats* mutant cells in mosaic discs overproliferate to form massive outgrowths that are sometimes larger than the mature discs themselves, and animals homozygous for many *lats* alleles also have dramatically overgrown discs (Figures 1A and F; Xu et al., 1995, Development 121:1053-1063). These *lats* phenotypes indicate that an inhibitory cell-cell communication mechanism has been disrupted and suggest that the lats protein could be a component of this mechanism regulating cell proliferation. The overproliferation phenotype of lats behaves in a cell autonomous fashion: inactivating lats causes mutant cells

to overproliferate (Xu et al., 1995, Development 121:1053-1063). Furthermore, in mosaic discs containing *lats* mutant clones, there is an overproliferation of *lats* mutant cells as well as a reduction in the number of wild type cell. These observations are consistent with a regulatory mechanism where *lats* mutant cells are able to send signals inhibiting cell proliferation but are defective in receiving such signals.

While the mammalian cdc2/cyclin A complex is involved in G2/M regulation (Hamaguchi et al., 1992, J. Cell Biol. 117:1041-1053; Hunter and Pines, 1994, Cell 79:573-582), *Drosophila* cdc2/cyclin A functions at the G1/S phase transition in addition to the G2/M phase transition. Ectopic activation of cdc2/cyclin A by overexpressing cyclin A in G1 arrested cells can drive the G1/S transition and induce S phase in cells lacking cyclin E (Dong et al., 1997, Genes & Devel. 11:94-105; Sprenger et al., 1997, Curr. Biol. 7:488-499). This G1/S activity is greatly enhanced when both cyclin A and an activated form of cdc2 are overexpressed. In *roughex (rux)* mutants, cells accumulate cyclin A in early G1 and progress into S phase precociously (Thomas et al., 1994, Cell 77:1003-1014; Thomas et al., 1997, Genes & Devel. 11:1289-1298). Loss of fizzy-related (fzr), a cdc2-related fly gene, results in accumulation of mitotic cyclins in G1 cells and causes progression through an extra division cycle in the embryonic epidermis (Sigrist and Lehner, 1997, Cell 90:671-681). These observations have shown that in *Drosophila* extra cdc2/Cyclin A activity can cause overproliferation. Consistent with these observations, we find that Cyclin A is abnormally accumulated in *lats* mutant cells (Figures 5A-N) and *lats* phenotypes can be suppressed by *cdc2* and *cyclin A* mutations. Several aspects of the *lats* phenotype are unique. First, *lats* mutants deregulate cdc2/cyclin A activities which affects both the G1/S and G2/M transitions. Second, while mutants such as *rux, fizzy(fzy)*, and *fzr* accumulate multiple mitotic cyclins and thus affect activities of several cdc2/cyclin complexes (Thomas et al., 1994, Cell 77:1003-1014; Dawson et al., 1995, J. Cell Biol. 129:725-737; Sigrist and Lehner, 1997, Cell 90:671-681), *lats* mutants appear to only affect cdc2/cyclin A. Finally, in *lats* mutant cells, cyclin A is degraded at late mitosis (Figure 5F), further indicating that many aspects of the cell cycle are normal in *lats* mutants. These properties distinguish *lats* mutants from genetic alterations that affect multiple CDK/cyclin complexes or that abnormally activate CDK/cyclin at a single cell-cycle stage or throughout the entire cell cycle, and provide an explanation for the extensive overproliferation phenotype of the *Drosophila lats* mutants.

The data provided herein indicate that cdc2/cyclin A activity is negatively regulated by the lats protein. Yeast two-hybrid assays show that lats specifically interacts with cdc2 but not other CDKs. Genetic data in *Drosophila* also show that lats interacts with cdc2 but not the fly CDK2 homolog, cdc2c. Given that p16- and p21-like CDK inhibitors have not been found for cdc2, it is possible that cdc2 and the rest of the CDKs are negatively regulated by different families of proteins. Alternatively, the activity of each CDK could be modulated by both types of negative regulators. In both flies and mammals, cdc2/cyclin A is inactivated during early mitosis by degradation of cyclin A, while degradation of cyclin B

occurs later at the metaphase/anaphase transition (Minshull et al., 1990, EMBO J. 9:2865-2875; Whitfield et al., 1990, EMBO J. 9:2563-2572). The mechanism of such differential inactivation of cdc2/cyclin is unknown. Our data indicate that lats specifically modulates cdc2/cyclin A activity but not cdc2/cyclin B activity: cyclin A but not cyclin B mutants interact with lats genetically; *lats* mutant cells abnormally accumulate cyclin A but not cyclin B (Figures 5A-N).

Finally, overexpression of cdc2 and cyclin A has been reported in multiple types of human tumors (Wang et al., 1990, Nature 343:555-557; Keyomarsi and Pardee, 1993, Proc. Natl. Acad. Sci. USA 90:1112-1116; Arany et al., 1994, Surg. Onc. 3:153-159). Negative regulators of CDK/Cyclins (*e.g.*, p16) have been shown to function as tumor suppressors in mammals (Serrano et al., 1996, Cell 85:27-37). The biochemical and genetic data for lats provided herein suggest that lats would behave as a tumor suppressor in mammals.

EXAMPLE 2: Mice Deficient for Lats Develop Soft Tissue Sarcomas, Ovarian Tumors and Pituitary Dysfunction

Materials and Methods

Generation of *lats*$^{-/-}$ mice

Mouse *lats* genomic DNA was isolated by screening a 129 library (Stratagene) using a mouse *lats* cDNA as a probe. A SalI fragment from the cDNA was subcloned into a pBS vector. We cleaved this construct at the EcoRV site (Figure 6B), and inserted a 1.8 kb fragment encoding PGK-neo. We subsequently digested with BamHI and XhoI and inserted a 3 kb PGK-TK gene cassette.

D3 embryonic stem (ES) cells were electroporated with the SfiI linearized vector, and selected in 0.3 mg/ml G418 and 2 μM ganciclovir media for incorporation of the vector. The ES cell clones analyzed underwent homologous recombination at the lats locus (Figure 6B). For genotyping, genomic DNA from the ES cells was digested with BamHI and EcoRV and analyzed by Southern blotting using the BamHI-EcoRI probe from the vector (Figure 6C). The double digest of the wild type allele generates a 3.5 kb fragment that hybridizes to the probe, while double digest of the disrupted allele generates a 5.8 kb fragment that hybridizes to the probe. *Lats* heterozygous ES cells were microinjected into CS7BL/6 blastocysts which were transplanted into uteri of pseudopregnant ICR mice. Chimeric male progeny were crossed to CS7BL/6 females. Germline transmission of the disrupted allele was detected in agouti progeny by Southern blotting.

Cell culture and protein analysis

Proteins were extracted from whole-cell lysates of *lats*$^{-/-}$ mouse embryonic fibroblasts (MEFs) derived from 13-days post-coitum mouse embryos, separated using SDS-PAGE, transferred and probed with rabbit polyclonal anti-lats antibody, followed by enhanced chemiluminescence detection (Amersham).

Histopathological examinations

For morphological evaluation, tissues were fixed in 10% neutral buffered formalin at 4°C overnight, dehydrated with ethanol, embedded in paraffin, and sectioned into 4 to 5 mm sections. Paraffin sections were prepared by standard procedures and stained with hematoxylin and eosin.

Gonadotropin treatment

Mice were injected intraperitoneally with FSH administered in the form of 5 IU of pregnant mare serum gonadotropin (Sigma). 44-46 hours later, mice were injected intraperitoneally with LH in the form of 5 IU of human chorionic gonadotropin (Sigma).

Pituitary hormone measurements

We used 20 *lats*$^{-/-}$ and 20 *lats*$^{+/-}$ age, sex, and estrus cycle matched females and males for these analyses. Mouse serum levels of PRL, LH, GH, FSH, and TSH were determined in pooled serum samples by double antibody radioimmunoassays (RIAs). These sensitive, specific mouse pituitary hormone RIAs were developed by A. F. Parlow, and are distributed to the scientific research community via the National Hormone & Pituitary Program of NIDDK, NIH (see http://www.humc.edu/hormones).

UVB and DMBA tumorigenic treatments

UVB and DMBA treatments were performed as described by Serrano et al. (1996, Cell 85:27-37). Briefly, skin tumors were induced by first applying a single dose of 9,10-dimethyl-1,2-benzathralene (DMBA; 50 µl of an 0.5% solution in acetone) to the dorsal surface of the mouse 1 to 5 days after birth. This treatment was followed by exposure to ultraviolet B (UVB) irradiation approximately three times per week for, on average, 27 treatments, with an initial exposure of approximately 100 mJ/cm2, increasing the dosage by 10% per treatment to a maximum of 700 mJ/cm2.

Results and Discussion

Targeted Disruption of the mouse *lats* gene

A 17.5 kilobase *lats* genomic clone obtained from a mouse 1295V library was used to construct a targeting vector for homologous recombination by positive-negative selection (Mansour et al., 1988, Nature 336:348-352; Capecchi, 1989, Science 244:1288-1292) as shown in Figure 6B. A PGK-neo cassette was inserted in inverse orientation into an exon of the lats clone resulting in the removal of amino acid sequence corresponding to amino acids 756-1130 of human lats (Figure 6A). We electroporated D3 embryonic stem cells (Gossler et al., 1986, Proc. Natl. Acad. Sci USA 83:9065-9069) with the lats-neo construct; single clones resistant to G418 and ganciclovir were screened by Southern blot hybridization using a mouse lats probe 5' to the portion of the *lats* gene contained in the targeting vector (Figure 6B). Restriction enzyme digestion of the wild-type lats locus with

BamHI and EcoRV generated a 3.5 kb fragment, while the correctly targeted, disrupted locus generated a 5.8 kb fragment (Figures 6B and C). A targeting frequency of approximately 1 in 100 was observed. Male chimeras transmitted the targeted *lats* allele through the germline, as demonstrated by Southern blot analysis of tail DNA. Immunoprecipitation and western blotting of *lats*$^{-/-}$ mouse embryonic fibroblast lysates (derived from 13.5 days post-coitum embryos (dpc)) with polyclonal anti-human lats antibody confirmed the absence of lats protein in homozygous null embryos (Figure 6D).

Growth and viability of *lats*$^{-/-}$ mice

When assessed at 3 weeks of age, the number of *lats* mutant animals was drastically lower than expected; 8% of pups genotyped from double heterozygote matings were *lats*$^{-/-}$, significantly lower than the expected frequency of 25%. Just before birth at embryonic day 18.5 (dpc), however, live homozygous embryos were found at the frequency predicted by Mendelian law (25%). The majority of homozygotes died within the first day of life, and their death was associated with internal hemorrhage into vital organs. The reason that some *lats*$^{-/-}$ mice survived while most did not is uncertain, but the mixed genetic background (strains 129 and C57BL/6) of the mice could be contributory. The weight of homozygous embryos at birth was approximately 70% of that of wild-type embryos (*lats*$^{+/+}$, 1.5 ± 0.3 g; *lats*$^{+/-}$, 1.3 ± 0.3 g; *lats*$^{-/-}$, 1.1 ± 0.1 g). All of these *lats*$^{-/-}$ mice were growth retarded. Most of the surviving *lats* homozygous mutant animals gained weight slowly, attaining only 60-70% of normal weight as adults (Figures 7A and B). A representative growth curve for lats deficient mice is shown in Figure 7B. To determine if there was a correlation between weight and growth, skeletal growth and organ weight was examined. By radiography, we observed differences in the skull and longitudinal bones that corresponded to the decrease in the size of the mouse. The decreased body weight was not due to the animals being leaner than their wild-type siblings, because the weight of most organs tested had decreased in proportion to the whole body weight. Exceptions were seen in a few particularly reduced organs, including the testis and the ovary.

Pituitary dysfunction

Male *lats*$^{-/-}$ mice displayed decreased fertility although histopathological examination of the testis did not reveal obvious structural abnormalities. *Lats*$^{-/-}$ females all displayed severe fertility defects, and approximately 60% of the females were completely sterile. Ovaries from all lats deficient females examined contained far fewer follicles than age and parity matched ovaries from *lats*$^{+/+}$ females (Figures 8A-D). The majority of follicles observed were primary and secondary follicles. Formation of the antrum was much less prominent than in normal mice. The follicles also contained fewer degenerative granulosa cells, which are common in atretic follicles in normal mice. Moreover, the formation of a corpus luteum was not detected (Figures 8A-D). These histological findings suggest an impairment of ovulation in *lats*$^{-/-}$ ovaries, and potential endocrine dysfunction.

The amount of breast epithelial tissue was markedly decreased in *lats*$^{-/-}$ females, with some females displaying a complete lack of macroscopic nipple-formation (Figures 9D and E). Histologically, the mammary glands of *lats*$^{-/-}$ mice were frequently reduced to a "fat pad" devoid of a ductular component (Figure 9F). This too suggested an endocrine component to the phenotype, as loss of lats may alter the levels of hormones that affect breast development.

Estrus is another indicator of endocrine function. Vaginal smears taken from control ($^{+/+}$) mice showed that they cycled through proestrus, estrus, metestrus, and diestrus in 4 days as described previously for normal mice (Nelson et al., 1982, Biol. Reprod. 27:327-339). In contrast, infertile *lats*$^{-/-}$ females did not cycle, and remained in continuous metestrus, an observation that further characterizes their infertility. The abnormal estrus cycle might reflect an underlying problem in signaling between the pituitary and the ovary. To determine if *lats*$^{-/-}$ ovaries could respond to appropriate gonadotropin stimulation, young adult mice (7 *lats*$^{-/-}$ females; 2 control females) were injected with a Follicle-Stimulating Hormone (FSH) analog (pregnant mare serum), and 46 hours later with a Luteinizing Hormone (LH) preparation (human chorionic gonadotropin). This treatment allowed *lats*$^{-/-}$ females to cycle into a prolonged period of estrus, confirming that the temporal synchronization of the levels of endogenous FSH and/or LH in *lats*$^{-/-}$ females is deficient.

In all *lats* knock-out mouse pituitaries examined (n=20), hyperplastic changes were readily apparent. There were multiple foci of atypical cells showing irregularly shaped nuclei with an increased content of chromatin and variability in size (Figures 10A and B). This histological atypia was accompanied by a pathological dysfunction of the pituitary. For example, hormone measurements in sera from *lats*$^{-/-}$ females revealed: the levels of LH were 3-fold lower than controls (Figure 10C); the PRL levels were less than half of controls (Figure 10D); and Growth Hormone (GH) levels were reduced by 25%. These deficiencies may have resulted from an unbalanced increase in the number of certain types of cells in this normally highly organized tissue. The reduced serum GH level may contribute to the reduced size of *lats*$^{-/-}$ mice. The diminished levels of serum LH could account for the lack of proper follicular maturation and differentiation, as well as the infertility observed in female *lats*$^{-/-}$ animals, with greater atypia in the pituitary leading to the more severe phenotype. The PRL and LH defects, together, account for both the lack of mammary gland development and the corpus luteum insufficiency syndrome which these animals display.

Interestingly, serum levels of pituitary Follicle Stimulating Hormone (FSH) (Figure 10E) and Thyroid Stimulating Hormone (TSH) are normal. The observation that *lats*$^{-/-}$ mice display a selective LH deficiency while sustaining normal FSH production supports a model of differential regulation of the two gonadotropins. This is in accordance with data from Lee and coworkers demonstrating that the transcription factor NGFI-A specifically regulates LH-ß (Lee et al., 1996, Science 273:1219-1221). *Lats*$^{-/-}$ mice thereby provide a model for the human reproductive dysfunction of isolated LH hypogonadotropic hypogonadism.

It is interesting to note that the pituitary deficiencies of *lats*$^{-/-}$ mice resembles those of other cell cycle regulator knock-out mice, such as the *Rb*$^{+/-}$, *p53*$^{-/-}$, and *p27*$^{-/-}$ mice. In these examples, pituitary cells and other endocrine organs appear to be crucially dependent on cell cycle regulation for their proper development. Indeed, tumor suppressors may play such a key role in the pituitary because critical function in this tissue allows for a link between control of single cell proliferation and total organismal growth and survival.

Tumor development in *lats*$^{-/-}$ mice

Another cause for the infertility of lats-deficient females is that they developed ovarian stromal cell tumors by the age of 3 months. The body of the normal ovary consists of spindle-shaped cells, reticular fibers and ground substance which together constitute the ovarian stroma, in which numerous follicles are embedded (Wheater et al., 1987, Functional Histology; Figure 8A). Stromal cell tumors in *lats*$^{-/-}$ mice obliterated the normal structure of the ovary, eliminating follicles progressively (Figures 8B and D). These stromal cell tumors are probably not resultant from pituitary dysfunction, as stromal cell tumors are most often local events (Clement, "Histology of the Ovary" in Histology For Pathologists, Second Ed., Sternberg, ed. (Lippincott-Raven, 1997) pp 934-935). Some *lats*$^{-/-}$ females were able to give birth to one litter, then became infertile as the stromal cell tumors expanded into the remaining functional ovary. To date, these stromal cell tumors have not yet displayed signs of malignancy. Stromal cell tumors were observed in all lats deficient mice examined (n=22), and extended throughout the entire ovary as determined by serial sectioning.

Additional evidence supporting the role of lats in mammalian tumorigenesis is that thus far, approximately 15% of all *lats*$^{-/-}$ females (n=28) between 4 to 10 months of age have developed large soft tissue sarcomas with metastases to vital organs (*e.g.* the lungs) (Figures 11A-C). Taking genetic backgrounds into consideration and using litter mates as controls, approximately 57% of all *lats*$^{-/-}$ animals developed soft tissue sarcomas. Histology revealed that these sarcomas consist of pleiomorphic, spindle-shaped cells with mitotic figures (Figure 11C), and they displayed immunohistochemical features of fibrosarcomas. No tumors were detected in control mice (n=80). It is possible that the spontaneous rate of tumor formation is even higher in *lats*$^{-/-}$ mice as some tumors may be occult, and therefore not readily identifiable. *Lats*$^{+/-}$ mice remained tumor free up to 8 months of age. This phenotype is consistent with that observed in heterozygotes for the CDK inhibitors, p27 and p16 (Fero et al., 1996, Cell 85:733-744; Kiyokawa et al., 1996, Cell 85:721-732; Nakayama et al., 1996, Cell 85:707-720; and Serrano et al., 1996, Cell 85:27-37).

The susceptibility of *lats*$^{-/-}$ mice to tumor induction by carcinogens was assessed using a standard two-stage protocol consisting of a single application of 9,10-dimethyl-1,2-benzanthracene (DMBA) followed by repeated exposure to ultraviolet B rays (Serrano et al., 1996, Cell 85:27-37). By 7 weeks of age, over 71% of *lats*$^{-/-}$ animals developed soft tissue sarcomas, whereas all of the control animals remained free of obvious tumors. The

frequency of tumor development in these *lats*$^{-/-}$ animals is particularly impressive, given the fact that C57BL/6 mice are poorly susceptible to the development of skin tumors (as reviewed by DiGiovanni, 1991, Pharm. & Ther. 54:63-128). The spontaneous and induced tumors observed in *lats*$^{-/-}$ animals provide clear evidence of the role of lats in mammalian tumorigenesis, and attest to the functional conservation of lats.

Although both *Drosophila* and mice develop tumors when lats is inactivated, the correlation between genotype and phenotype differs between the two organisms. While every *lats*$^{-/-}$ cell overproliferates in mosaic flies (Xu et al. 1995, Development 121:1053-1063), only certain tissues in *lats*$^{-/-}$ mice develop tumors. This phenotypic difference could be due to the increased complexity in cell cycle control in mammals. For example, in *Drosophila*, cdc2/Cyclin A functions at both the G1/S and G2/M transitions in the cell cycle (Whitfield et al., 1990, EMBO J. 9:2563-2573; Knoblich and Lehner, 1993, EMBO J. 12:65-74; Knoblich et al., 1994, Cell 77:117-120; Sauer et al., 1995, Genes & Devel. 9:1237-1239; Sigrist and Lehner, 1997, Cell 90:671-681; Sprenger et al., 1997, Curr. Biol. 7:488-499; Thomas et al., 1997, Genes & Dev. 11:1289-1298). In mammals, however, a different CDK, CDK2, complexes with cyclin A to regulate the G1/S transition, while cdc2/cyclin A functions in the G2/M transition (Girard et al., 1991, Cell 67:1169-1179; Tsai et al., 1991, Nature 353:174-177; Hamaguchi et al., 1992, J. Cell. Biol. 117:1041-1053; Pagano et al., 1992, EMBO J. 11:961-967; Rosenblatt et al., 1992, Proc. Natl. Acad. Sci. USA 89:2824-2828; Hunter and Pines, 1994, Cell 79:573-582; Resnitzky et al., 1995, Mol. Cell Biol. 15:4347-4352). We have shown that lats negatively regulates cdc2/cyclin A, but does not appear to interact with CDK2. Thus, while inactivation of *lats* in *Drosophila* affects regulation of both the G1/S and G2/M transitions of the cell cycle, the removal of lats function in mammalian cells only affects control of the G2/M transition. This result is consistent with the fact that human cancers are often caused by mutations in multiple, non-homologous cell cycle control pathways (Kinzler and Vogelstein, 1996, Cell 87:159-170; Weinberg, 1996, Sci. Am. 275:62-70). The higher degree of redundancy in mammalian genomes could also contribute to the phenotypic difference between fly and mouse *lats* mutants.

Although most human tumor suppressors that have been characterized function in the regulation of G1/S (Brown, 1997, Adv. Genet. 36:45-135), there are indications that deregulation of G2/M and M controls may also contribute to tumorigenesis in mammals. For example, p53 is involved in the regulation of both G1/S and G2/M (and M) (Cross et al., 1995, Science 267:1353-1356; Hermeking et al., 1997, Mol. Cell 1:3-11). Inactivation of p53 in mice leads to impressive tumor development, while the disruption of p21, the p53-downstream effector for G1/S, has no tumorigenic effect (Deng et al., 1995, Cell 82:675-684). Recent data also suggests that Rb plays a role in G2/M in addition to its G1/S involvement (Niculescu et al., 1998, Mol. Cell Biol. 18:629-643). Furthermore, overexpression of cdc2 and mitotic cyclins has been reported in multiple types of human tumors (Arany et al., 1994, Surg, Oncol. 3:153-159; Keyomarsi and Pardee, 1993, Proc.

Natl. Acad. Sci USA 90:1112-1116; Wang et al., 1990, Nature 343:555-557). Signaling through the RHAMM receptor affects cell proliferation by down-regulation of cdc2 and cyclin B transcripts and proteins and results in reversal of tumorigenesis (Mohapatra et al., 1996, J. Exper. Med. 183:1663-1668). The tumorigenic phenotype of *lats*$^{-/-}$ mice further supports the notion that the regulation of G2/M or M also plays a role in mammalian tumor development.

A comparison between lats- and p16-knock-out mice is interesting in several respects. Both lats and p16 are negative regulators of CDKs. In addition, lats-knock-out mice resemble p16-knock-out animals (Serrano et al., 1996, Cell 85:27-37) in that homozygotes develop tumors at an early age while heterozygotes do not. Although different types of tumors are observed in these two mutants (*e.g.*, ovarian tumors in *lats*$^{-/-}$ mice and lymphomas in *p16*$^{-/-}$ mice), both types of knock-out mice develop soft tissue sarcomas. The frequencies of spontaneous soft tissue sarcomas between these two knock-out mutants cannot be directly compared due to protocol differences, however, induced tumors in these animals were obtained using the same induction protocol (Serrano et al., 1996, Cell 85:27-37). Interestingly, the frequency of induced tumor formation in *lats*-knock-out mice is even higher than that observed for *p16*$^{-/-}$ mice. Over 71% of *lats*$^{-/-}$ animals developed soft tissue sarcomas by 7 weeks of age. Only 10% of the *p16*$^{-/-}$ mice developed tumors by 7 weeks of age, and 60% of them displayed tumors between the ages of 6-12 weeks (Serrano et al., 1996, Cell 85:27-37).

The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and accompanying figures. Such modifications are intended to fall within the scope of the appended claims.

Various references are cited herein, the disclosures of which are incorporated by reference in their entireties.

WHAT IS CLAIMED IS:

1. A recombinant non-human animal in which a *lats* gene has been inactivated by a method comprising introducing a nucleic acid into the animal, or an ancestor thereof, which nucleic acid comprises a non-*lats* sequence flanked by *lats* genomic sequences that promote homologous recombination, such that said non-*lats* sequence replaces the nucleotide sequence encoding the Lats C-terminal domain 1, the Lats C-terminal domain 2, the Lats C-terminal domain 3, and a portion of the kinase domain of the lats protein encoded by the *lats* gene.

2. The recombinant non-human animal of claim 1 in which the non-*lats* sequence replaces the nucleotide sequence encoding the amino acids that correspond to amino acids 756-1130 of human lats, as depicted in Figure 12 (SEQ ID NO:2).

3. The recombinant non-human animal of claim 1 which is a mouse.

4. The recombinant non-human animal of claim 3 in which the *lats* gene contains a lats coding sequence of SEQ ID NO:3.

5. The recombinant non-human animal of claim 1 in which both alleles of the *lats* gene have been inactivated.

6. A method for screening a potential therapeutic compound for activity in treating or preventing cancer comprising administering the compound to the recombinant non-human animal of claim 1; and comparing the size or progression of the cancer in the recombinant non-human animal to which the compound was administered with the size or progression of the cancer in the same recombinant non-human animal prior to administration of the compound or in a recombinant non-human animal that was not so administered or to a standard size or progression of the cancer for such same or a recombinant non-human animal that was not so administered, wherein a decrease in the size or progression of the cancer in the recombinant non-human animal administered the compound as compared to the same animal prior to the administration or to the recombinant non-human animal not so administered or to the standard size or progression of the cancer, indicates that the compound has activity in treating or preventing cancer.

7. The method of claim 6 in which the non-*lats* sequence replaces the nucleotide sequence encoding the amino acids that correspond to amino acids 756-1130 of human lats, as depicted in Figure 12 (SEQ ID NO:2).

8. The method of claim 6 in which the recombinant non-human animal is a mouse.

9. The method of claim 8 in which the *lats* gene contains the lats coding sequence of SEQ ID NO:3.

10. The method of claim 6 in which both alleles of the *lats* gene have been inactivated.

11. The method of claim 6 in which the compound is screened for activity in treating or preventing soft tissue sarcomas.

12. The method of claim 6 in which the compound is screened for activity in treating or preventing ovarian tumors.

13. A method for screening a potential therapeutic compound for activity in treating or preventing cancer comprising recombinantly expressing the compound in the recombinant non-human animal of claim 1; and comparing the size or progression of the cancer in the recombinant non-human animal in which the compound was expressed with the size or progression of the cancer in the same recombinant non-human animal prior to expression of the compound or in a recombinant non-human animal in which the compound was not so expressed or to a standard size or progression of the cancer for such same or a recombinant non-human animal in which the compound was not so expressed, wherein a decrease in the size or progression of the cancer in the recombinant non-human animal in which the compound was expressed as compared to the same animal prior to the expression of the compound or to the recombinant non-human animal in which said compound was not so expressed or to the standard size or progression of the cancer, indicates that the compound has activity in treating or preventing cancer.

14. A method for screening a potential therapeutic compound for activity in treating or preventing skin cancer comprising administering the compound to a *lats* knock-out animal having skin tumors induced by exposure to at least one carcinogen; and comparing the size or progression of the skin tumors on the *lats* knock-out animal to which the compound was administered with the size or progression of skin cancers on the same *lats* knock-out animal prior to administration of the compound or on a *lats* knock-out animal in which skin tumors have also been induced by exposure to said at least one carcinogen but which has not been administered the compound or to a standard size or progression of the skin tumors for such same or a *lats* knock-out animal that was not so administered, wherein a reduction in the size or progression of the skin tumors in the *lats* knock-out animal administered the compound as compared to the same animal prior to

administration of the compound or to the animal not so administered or to the standard size or progression of the skin tumors, indicates that the compound has activity in treating or preventing skin cancer.

15. The method of claim 14 in which the *lats* knock-out animal has at least one *lats* gene which was inactivated by promoting homologous recombination between *lats* genomic sequences and a nucleic acid having non-*lats* sequences flanked by genomic sequences.

16. The method of claim 15 in which the non-*lats* sequence replaces the nucleotide sequence encoding the Lats C-terminal domain 1, the Lats C-terminal domain 2, the Lats C-terminal domain 3, and a portion of the kinase domain of the lats protein encoded by the *lats* gene.

17. The method of claim 15 in which the non-*lats* sequence replaces the nucleotide sequence1 encoding the amino acids that correspond to amino acids 756-1130 of human lats, as depicted in Figure 12 (SEQ ID NO:2).

18. The method of claim 14 in which the *lats* knock-out animal is a mouse.

19. The method of claim 18 in which the *lats* gene contains the lats coding sequence of SEQ ID NO:3.

20. The method of claim 14 in which both alleles of the *lats* gene have been inactivated.

21. The method of claim 14 in which the skin tumors were induced by 9,10-dimethyl-1,2-benzanthracene and repeated exposure to ultraviolet B radiation.

22. The method of claim 14 in which the potential therapeutic compound is administered topically.

23. A method for screening a potential therapeutic compound for activity in treating or preventing skin cancer comprising recombinantly expressing the compound in a *lats* knock-out animal having skin tumors induced by exposure to at least one carcinogen; and comparing the size or progression of the skin tumors on the *lats* knock-out animal in which the compound was expressed with the size or progression of skin cancers on the same *lats* knock-out animal prior to expression of the compound or on a *lats* knock-out animal in which skin tumors have also been induced by exposure to said at least one carcinogen but in which the compound has not been expressed or to a standard size or progression of the skin

tumors for such same or a *lats* knock-out animal in which the compound was not so expressed, wherein a reduction in the size or progression of the skin tumors in the *lats* knock-out animal in which the compound was expressed as compared to the same animal prior to expression of the compound or to the animal in which the compound was not so expressed or to the standard size or progression of the skin tumors, indicates that the compound has activity in treating or preventing skin cancer.

24. A method for screening a potential therapeutic compound for activity in treating or preventing a disease or disorder associated with pituitary dysfunction comprising administering the compound to a *lats* knock-out animal; and comparing the level of an indicator of pituitary dysfunction in the *lats* knock-out animal to which the compound has been administered to the level of the indicator in the same *lats* knock-out animal prior to administration of the compound or to a *lats* knock-out animal that has not been administered the compound or to a standard level of the indicator for such same or a *lats* knock-out animal that was not so administered, wherein a change in the indicator toward the level of the indicator in a wild type animal not afflicted with a pituitary dysfunction as compared to the same animal prior to administration of the compound or to the animal not so administered or to the standard level of the indicator, indicates that the compound is active to treat or prevent a disease or disorder associated with pituitary dysfunction.

25. The method of claim 24 in which the *lats* knock-out animal has at least one *lats* gene which was inactivated by promoting homologous recombination between *lats* genomic sequences and a nucleic acid having non-*lats* sequences flanked by genomic sequences.

26. The method of claim 25 in which the non-lats sequence replaces the nucleotide sequence encoding the Lats C-terminal domain 1, the Lats C-terminal domain 2, the Lats C-terminal domain 3, and a portion of the kinase domain of the lats protein encoded by the *lats* gene.

27. The method of claim 25 in which the non-*lats* sequence replaces the nucleotide sequence encoding the amino acids that correspond to amino acids 756-1130 of human lats, as depicted in Figure 12 (SEQ ID NO:2).

28. The method of claim 24 in which the *lats* knock-out animal is a mouse.

29. The method of claim 28 in which the *lats* gene contains a lats coding sequence of SEQ ID NO:3.

30. The method of claim 24 in which both alleles of the *lats* gene have been inactivated.

31. The method of claim 24 in which the indicator is fertility.

32. The method of claim 24 in which the indicator is ovulation.

33. The method of claim 24 in which the indicator is linear growth.

34. The method of claim 24 in which the indicator is serum levels of luteinizing hormone, growth hormone or prolactin.

35. The method of claim 24 in which the disease or disorder is LH hypogonadotropic hypogonadism.

36. A method for screening a potential therapeutic compound for activity in treating or preventing a disease or disorder associated with pituitary dysfunction comprising recombinantly expressing the compound in a *lats* knock-out animal; and comparing the level of an indicator of pituitary dysfunction in the *lats* knock-out animal in which the compound has been expressed to the level of the indicator either in the same *lats* knock-out animal prior to expression of the compound or to a *lats* knock-out animal in which the compound has not been expressed or to a standard level of the indicator for such same or a *lats* knock-out animal in which the compound was not so expressed, wherein a change in the indicator toward the level of the indicator in a wild type animal not afflicted with a pituitary dysfunction as compared to the same animal prior to expression of the compound or to the animal in which the compound was not so expressed or to the standard level of the indicator, indicates that the compound is active to treat or prevent a disease or disorder associated with pituitary dysfunction.

37. The method of claim 6, 14 or 24 in which the compound is purified.

38. A method for treating a cancer that has been shown to be refractory to a chemotherapy or radiation therapy in a subject in need of such treatment comprising administering to the subject a therapeutically effective amount of a molecule that promotes lats function.

39. The method of claim 38 in which the subject is a human.

40. The method of claim 38 in which the molecule is a lats protein.

41. The method of claim 38 in which the molecule is a protein having an amino acid sequence selected from the group consisting of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, and SEQ ID NO:8.

42. The method of claim 38 in which the molecule is a protein having the amino acid sequence of SEQ ID NO:2.

43. The method of claim 38 in which the molecule is a lats analog or derivative that has activity to promote lats function.

44. The method of claim 38 in which the molecule is a protein encoded by a first nucleic acid that is hybridizable under conditions of low stringency to a second nucleic acid having a nucleotide sequence that is the reverse complement of a sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, and SEQ ID NO:7, said protein having activity to inhibit cell overproliferation.

45. The method of claim 38 in which the molecule is a protein consisting of at least 20 contiguous amino acids of a protein having an amino acid sequence selected from the group consisting of SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6, and SEQ ID NO:8, said protein having activity to inhibit cell overproliferation.

46. The method of claim 38 in which the molecule is a protein comprising a domain of a lats protein selected from the group consisting of a lats C-terminal domain 3 (LCD3), lats C-terminal domain 2 (LCD2), lats C-terminal domain 1 (LCD1), kinase domain, lats flanking domain (LFD), lats split domain 1 (LSD1), lats split domain 2 (LSD2), and SH3-binding domain, said protein having activity to inhibit cell overproliferation.

47. The method of claim 40 in which the lats protein is phosphorylated.

48. The method of claim 47 in which the lats protein is phosphorylated on a serine or threonine residue within 20 residues upstream of the amino acid sequence Ala-Pro-Glu in a subdomain eight of a kinase domain of said lats protein.

49. The method of claim 48 in which the lats protein is phosphorylated at a serine residue corresponding to serine 909 of the human lats amino acid sequence, as depicted in Figure 12 (SEQ ID NO:2).

50. The method of claim 43 in which the lats analog or derivative has a threonine or serine residue within 20 residues upstream of the amino acid sequence Ala-Pro-Glu in a

subdomain eight of a kinase domain of said lats analog or derivative substituted with an aspartate or glutamate residue.

51. The method of claim 50 in which the lats analog or derivative has a glutamate residue substituted for a serine residue at the residue corresponding to serine 909 of the human lats amino acid sequence, as depicted in Figure 12 (SEQ ID NO:2).

52. The method of claim 38 in which said molecule is a chimeric protein comprising a fragment of a lats protein, said fragment consisting of at least 20 contiguous amino acids of said lats protein, fused via a covalent bond to an amino acid sequence of a second protein, said second protein not being a lats protein, said chimeric protein having activity to inhibit cell overproliferation.

53. The method of claim 38 in which said cancer has been shown to be refractory to radiation therapy.

54. The method of claim 38 in which said cancer has been shown to be refractory to chemotherapy.

55. The method of claim 54 in which said chemotherapy kills cancer cells during S phase of the cell cycle.

56. The method of claim 54 in which said chemotherapy kills cancer cells during mitosis.

57. The method of claim 38 which further comprises administering one or more chemotherapeutic agents to the subject.

58. The method of claim 57 in which said one or more chemotherapeutic agents are administered concurrently with the administration of said molecule.

59. The method of claim 57 in which said one or more chemotherapeutic agents are administered subsequent to the administration of said molecule.

60. The method of claim 38 in which said molecule is a nucleic acid comprising a nucleotide sequence encoding a lats protein.

61. The method of claim 60 in which said nucleotide sequence is SEQ ID NO:1.

62. The method of claim 60 in which said nucleotide sequence encodes a protein having the amino acid sequence of SEQ ID NO:2.

63. The method of claim 38 in which said molecule is a first nucleic acid that hybridizes under low stringency conditions to a second nucleic acid that is the reverse complement of a sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, and SEQ ID NO:7.

64. A method for treating a cancer that has been shown to be refractory to a chemotherapy or radiation therapy in a subject in need of such treatment comprising administering to the subject a therapeutically effective amount of a cell that expresses a recombinant nucleic acid that promotes lats function.

65. The method of claim 64 in which said nucleic acid comprises the nucleotide sequence of SEQ ID NO:1.

66. The method of claim 64 in which said nucleic acid comprises a nucleotide sequence encoding a protein having the amino acid sequence of SEQ ID NO:2.

67. A kit comprising in one or more containers a therapeutically effective amount of a molecule selected from the group consisting of a lats protein, a lats derivative, a lats analog, a nucleic acid encoding a lats protein, a nucleic acid encoding a lats derivative, and a nucleic acid encoding a lats analog; and at least one chemotherapeutic agent.

68. A purified complex of a lats protein and a cdc2 protein.

69. The purified complex of claim 68 in which the proteins are human proteins.

70. The purified complex of claim 68 in which the lats protein is phosphorylated.

71. The purified complex of claim 70 in which the lats protein is phosphorylated on a serine or threonine residue within 20 residues upstream of the amino acid sequence Ala-Pro-Glu in a subdomain eight of a kinase domain of said lats protein.

72. The purified complex of claim 71 in which the lats protein is phosphorylated at a serine residue corresponding to serine 909 of the human lats amino acid sequence, as depicted in Figure 12 (SEQ ID NO:2).

73. A purified complex selected from the group consisting of a complex of a derivative of a lats and a cdc2 protein, a complex of a lats protein and a derivative of a cdc2,

and a complex of a derivative of a lats protein and a derivative of a cdc2 protein, in which the derivative of the lats protein is able to form a complex with a wild-type cdc2 protein and the derivative of the cdc2 is able to form a complex with a wild-type lats protein.

74. The purified complex of claim 73 in which the derivative of the lats protein and/or the cdc2 protein is fluorescently labeled.

75. The purified complex of claim 73 in which the lats derivative has a threonine or serine residue within 20 residues upstream of the amino acid sequence Ala-Pro-Glu in a subdomain eight of a kinase domain of said lats derivative substituted with an aspartate or glutamate residue.

76. The purified complex of claim 75 in which the lats derivative has a glutamate residue substituted for a serine residue at the residue corresponding to serine 909 of the human lats amino acid sequence, as depicted in Figure 12 (SEQ ID NO:2).

77. The purified complex of claim 73 in which the lats derivative is a fragment of a lats protein consisting of the amino acid sequence corresponding to amino acids 15-585 of the amino acid sequence of human lats, as depicted in Figure 12 (SEQ ID NO:2).

78. A chimeric protein comprising a fragment of a lats protein consisting of at least 6 amino acids fused via a covalent bond to a fragment of a cdc2 protein consisting of at least 6 amino acids.

79. The chimeric protein of claim 78 in which the fragment of the lats protein is a fragment capable of binding the cdc2 protein and in which the fragment of the cdc2 protein is a fragment capable of binding the lats protein.

80. The chimeric protein of claim 78 in which the fragment of the lats protein has an amino acid sequence corresponding to amino acids 15 to 585 of the amino acid sequence of human lats, as depicted in Figure 12 (SEQ ID NO:2).

81. The chimeric protein of claim 79 in which the fragment of the lats protein and the fragment of the cdc2 protein form a lats-cdc2 complex.

82. An antibody which immunospecifically binds the complex of claim 68 or a fragment or derivative of said antibody containing the binding domain thereof.

83. The antibody of claim 82 which does not immunospecifically bind a lats protein or a cdc2 protein that are not part of a lats-cdc2 complex.

84. An isolated nucleic acid or an isolated combination of nucleic acids comprising a nucleotide sequence encoding a lats protein and a nucleotide sequence encoding a cdc2 protein.

85. The isolated nucleic acid or isolated combination of nucleic acids of claim 84 which are nucleic acid vectors.

86. An isolated nucleic acid that comprises a nucleotide sequence encoding the chimeric protein of claim 78.

87. A cell containing the nucleic acid of claim 84, which nucleic acid is recombinant.

88. A cell containing the nucleic acid of claim 86, which nucleic acid is recombinant.

89. A pharmaceutical composition comprising a therapeutically or prophylactically effective amount of the complex of claim 68; and a pharmaceutically acceptable carrier.

90. The pharmaceutical composition of claim 89 in which the proteins are human proteins.

91. A pharmaceutical composition comprising a therapeutically or prophylactically effective amount of the complex of claim 73; and a pharmaceutically acceptable carrier.

92. A pharmaceutical composition comprising a therapeutically or prophylactically effective amount of the chimeric protein of claim 79; and a pharmaceutically acceptable carrier.

93. A pharmaceutical composition comprising a therapeutically or prophylactically effective amount of the antibody of claim 83 or a fragment or derivative of said antibody containing the binding domain thereof; and a pharmaceutically acceptable carrier.

94. A pharmaceutical composition comprising a therapeutically or prophylactically effective amount of the nucleic acids or combination of nucleic acids of claim 84; and a pharmaceutically acceptable carrier.

95. A pharmaceutical composition comprising a therapeutically or prophylactically effective amount of the isolated nucleic acid of claim 86; and a pharmaceutically acceptable carrier.

96. A pharmaceutical composition comprising a therapeutically or prophylactically effective amount of the recombinant cell of claim 87; and a pharmaceutically acceptable carrier.

97. A pharmaceutical composition comprising a therapeutically or prophylactically effective amount of the recombinant cell of claim 88; and a pharmaceutically acceptable carrier.

98. A method of producing a complex of a lats protein and a cdc2 protein comprising growing a recombinant cell containing the nucleic acid of claim 84 such that the encoded lats and cdc2 proteins are expressed and bind to each other, and recovering the expressed complex of the lats protein and the cdc2 protein.

99. A method of diagnosing or screening for the presence of or a predisposition for developing a disease or disorder characterized by an aberrant level of a complex of a lats protein and a cdc2 protein in a subject comprising measuring the level of said complex, RNA encoding the lats and the cdc2 proteins, or functional activity of said complex, in a sample derived from the subject, in which an increase or decrease in the level of said complex, said RNA encoding lats and cdc2, or functional activity of said complex in the sample, relative to the level of said complex, said RNA encoding lats and cdc2, or functional activity of said complex found in an analogous sample from a subject not having the disease or disorder or a predisposition for developing the disease or disorder, indicates the presence of the disease or disorder or a predisposition for developing the disease or disorder.

100. A kit comprising in one or more containers a substance selected from the group consisting of a complex of a lats and a cdc2 protein, an antibody against said complex, nucleic acid probes capable of hybridizing to RNA of lats and RNA of cdc2, or pairs of nucleic acid primers capable of priming amplification of at least a portion of a gene for lats and a gene for cdc2.

101. A method for modulating the activity of cdc2 comprising administering a molecule that promotes, inhibits, or antagonizes lats function.

102. A method for inhibiting the activity of cdc2 comprising administrating a molecule that promotes lats function.

103. A method for increasing the activity of cdc2 comprising administering a molecule that inhibits or antagonizes lats function.

104. A method for treating or preventing a disease or disorder associated with an aberrantly high level of cdc2 in a subject in need of such treatment or prevention comprising administering to the subject a therapeutically effective amount of a molecule that promotes lats function.

105. The method of claim 104 in which said molecule is selected from the group consisting of a lats protein, a lats derivative or analog that promotes lats function, a nucleic acid encoding a lats protein, and nucleic acid encoding a lats derivative or analog that promotes lats function, and a lats agonist.

106. A method for treating or preventing a disease or disorder associated with an aberrantly low level of cdc2 activity in a subject in which such treatment or prevention is desired comprising administering to the subject a therapeutically effective amount of a molecule that inhibits or antagonizes lats function.

107. The method of claim 106 in which said molecule is selected from the group consisting of a lats analog or derivative that inhibits or antagonizes lats function, an anti-lats antibody, and a *lats* antisense nucleic acid.

108. A method for screening a molecule for efficacy in treating or preventing a cancer refractory to chemotherapy or radiation therapy, said method comprising contacting cancer cells that are refractory to treatment with chemotherapeutic agents or radiation with the molecule and comparing the proliferation or survival of the contacted cells with the proliferation or survival of cells not so contacted, wherein a lower level of proliferation or survival of the contacted cells indicates that the molecule is effective to treat or prevent the cancer.

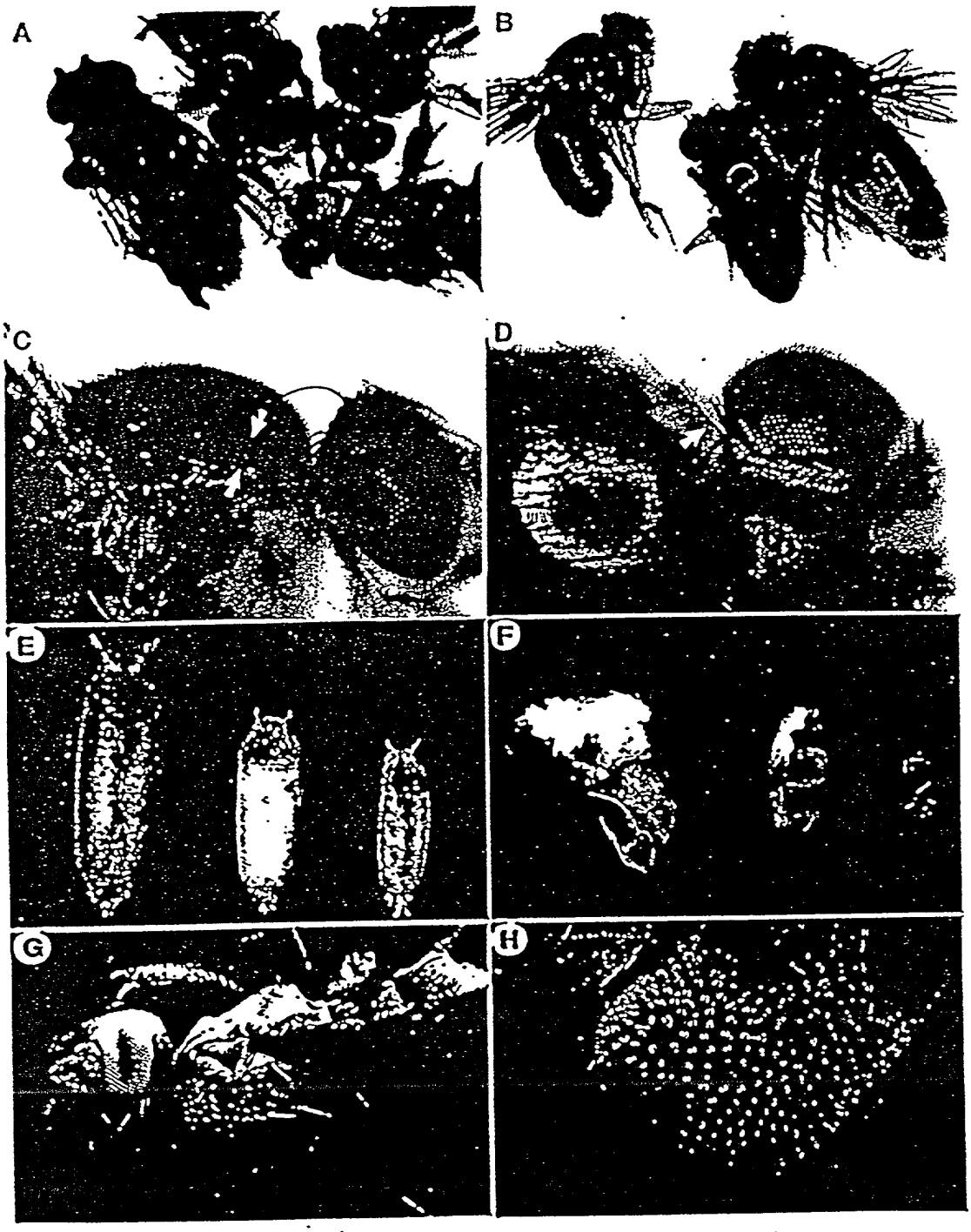
109. The method of claim 108 in which said cells are cultured *in vitro* from a tissue sample of a patient.

110. A method for screening a molecule for activity to modulate cdc2 levels or activity comprising contacting cells with the molecule, and comparing the level of cdc2 protein, mRNA or activity in cells contacted with the molecule to the amount of cdc2 protein, mRNA, or activity in cells not so contacted, wherein an increase or decrease in the amount of cdc2 protein, mRNA, or activity in the contacted cells relative to the amount of cdc2 protein, mRNA, or activity in the cells not so contacted indicates that the molecule has activity to modulate cdc2 levels or activity.

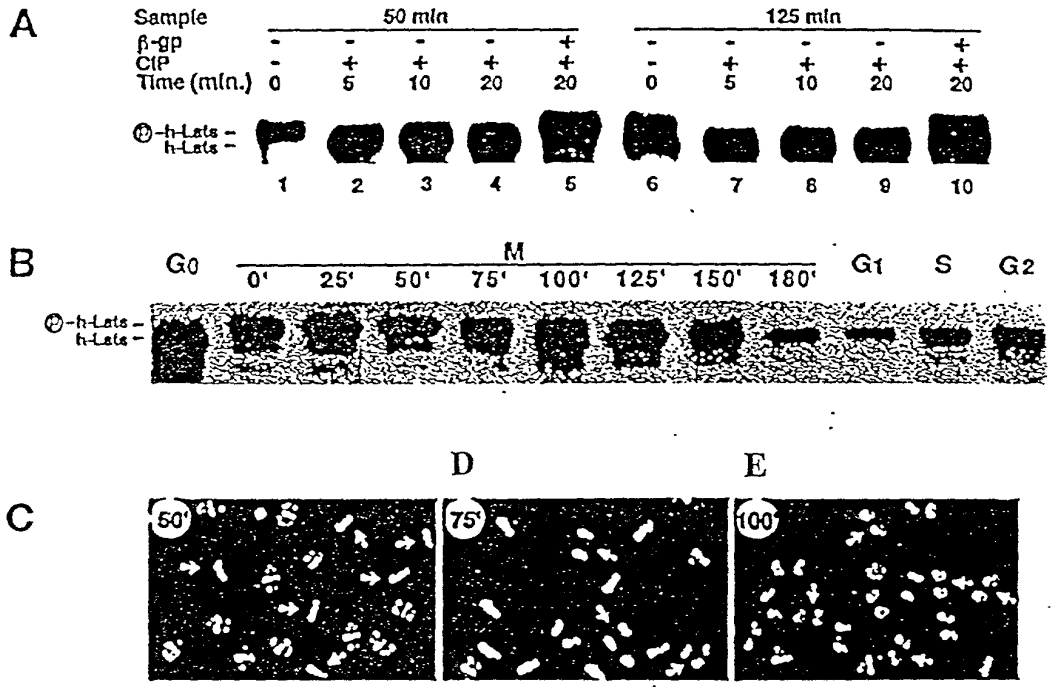
111. A method for screening a molecule for activity to modulate, directly or indirectly, the formation of a complex of lats and cdc2 proteins comprising measuring the levels of said complex formed from lats and cdc2 proteins in the presence of said molecule under conditions conducive to formation of the complex; and comparing the levels of said complex with the levels of said complex that are formed in the absence of said molecule, wherein a lower or higher level of said complex in the presence of said molecule indicates that the molecule modulates formation of said complex.

112. The method of claim 111 in which the molecule inhibits formation of the complex.

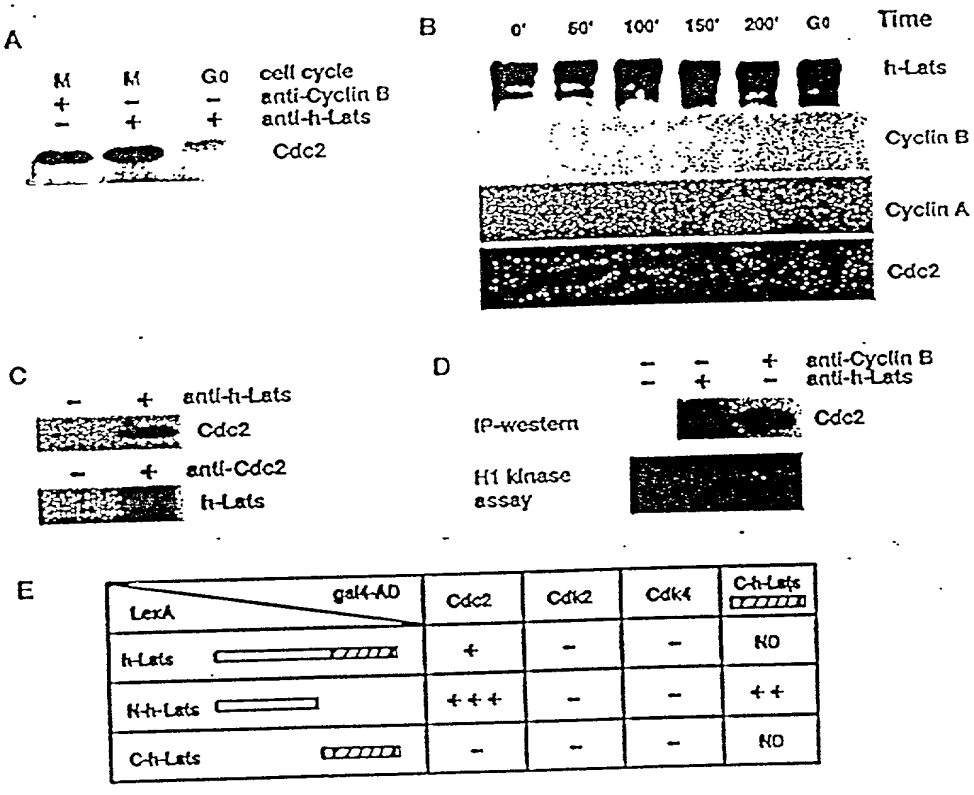
113. The method of claim 111 in which the molecule promotes formation of the complex.



FIGS. 1A-H


A
Sample
50 min
125 min
β-gp
CIP
Time (min.)
0
5
10
20
20
0
5
10
20
20
P-h-Lats
h-Lats
1
2
3
4
5
6
7
8
9
10
B
G0
M
G1
S
G2
0'
25'
50'
75'
100'
125'
150'
180'
P-h-Lats
h-Lats
C
D
E
50'
75'
100'

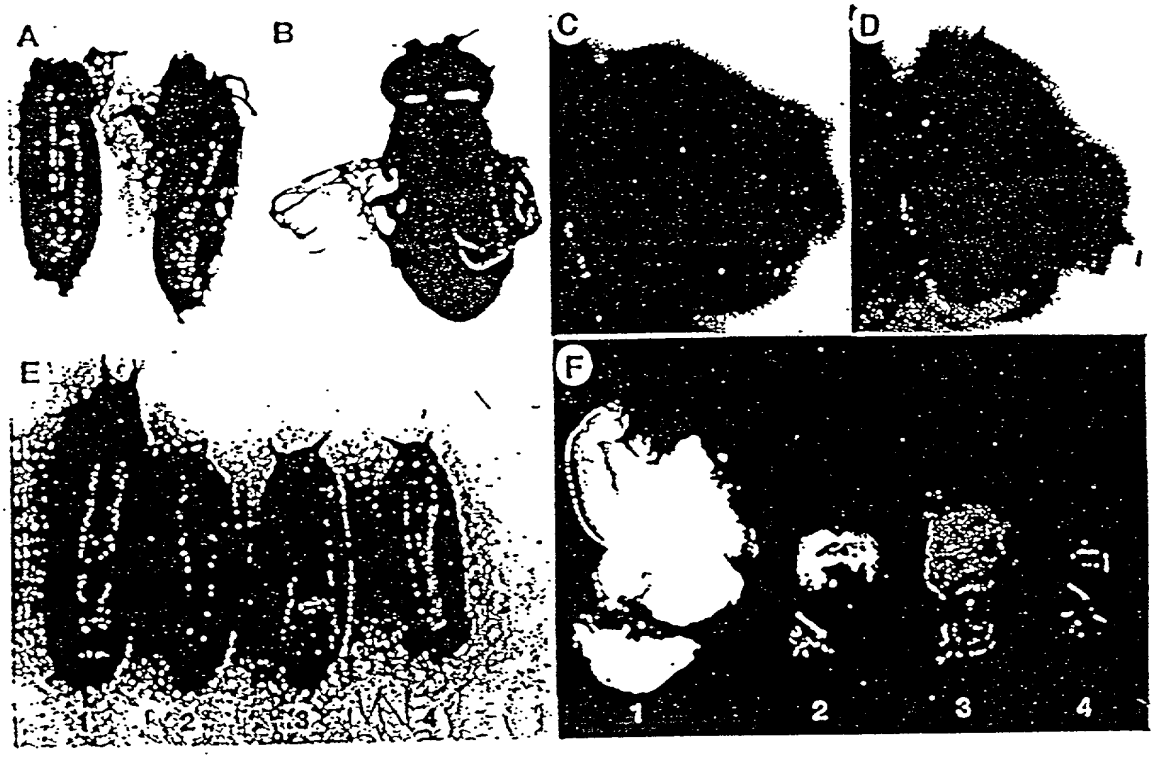
FIGS. 2A-E



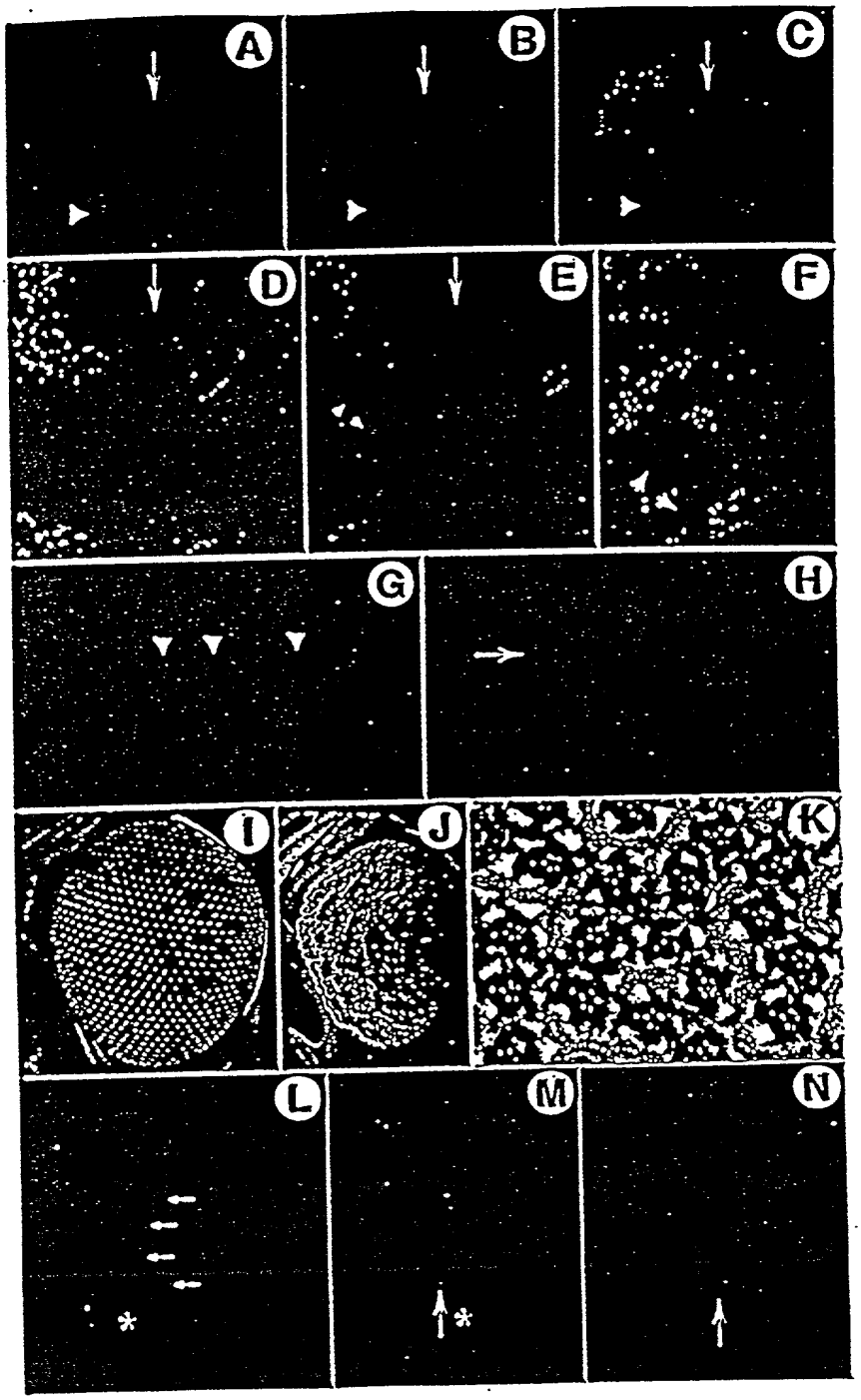
E

LexA \ gal4-AD	Cdc2	Cdk2	Cdk4	C-h-Lats
h-Lats	+	–	–	ND
N-h-Lats	+ + +	–	–	+ +
C-h-Lats	–	–	–	ND

FIGS. 3A-E



FIGS. 4A-F



FIGS. 5A-N

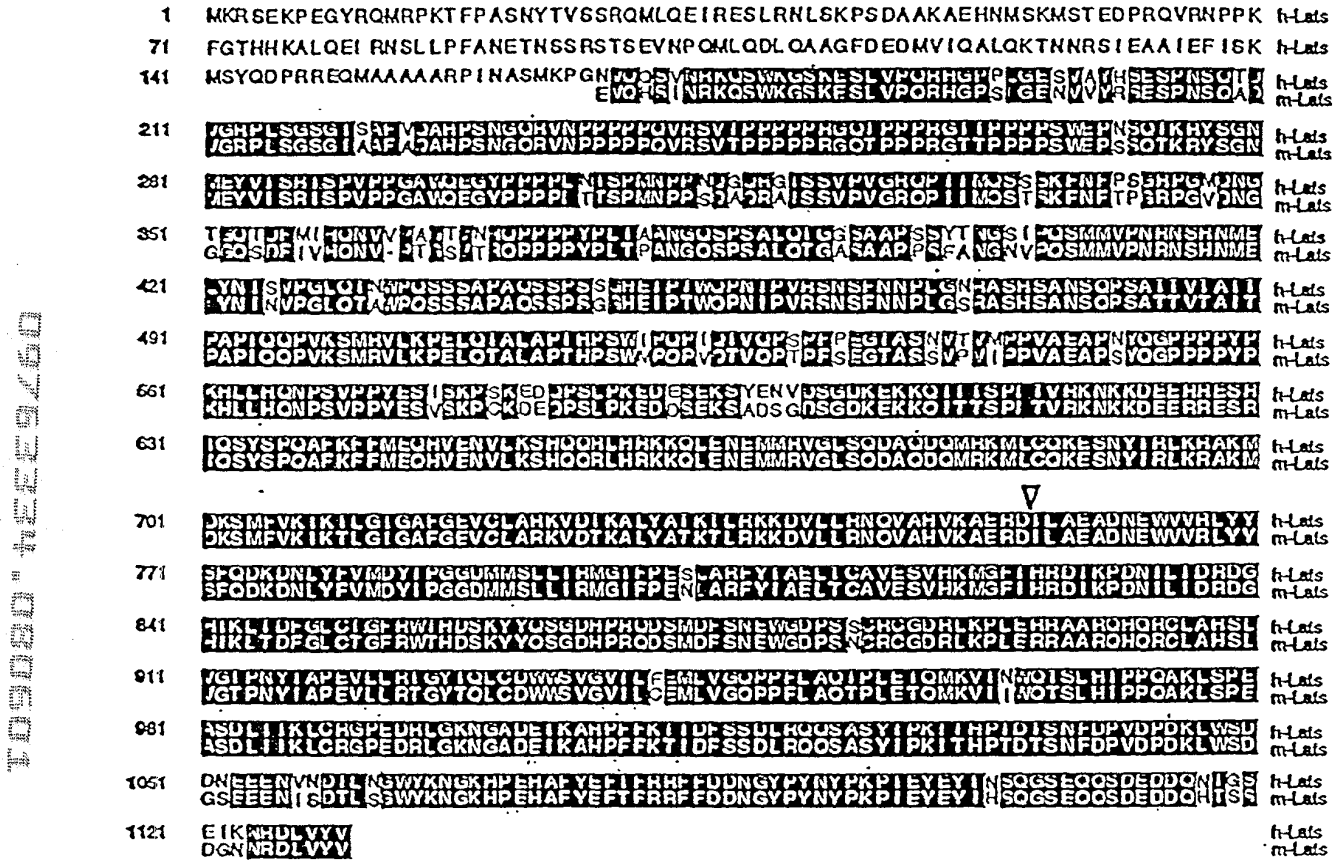
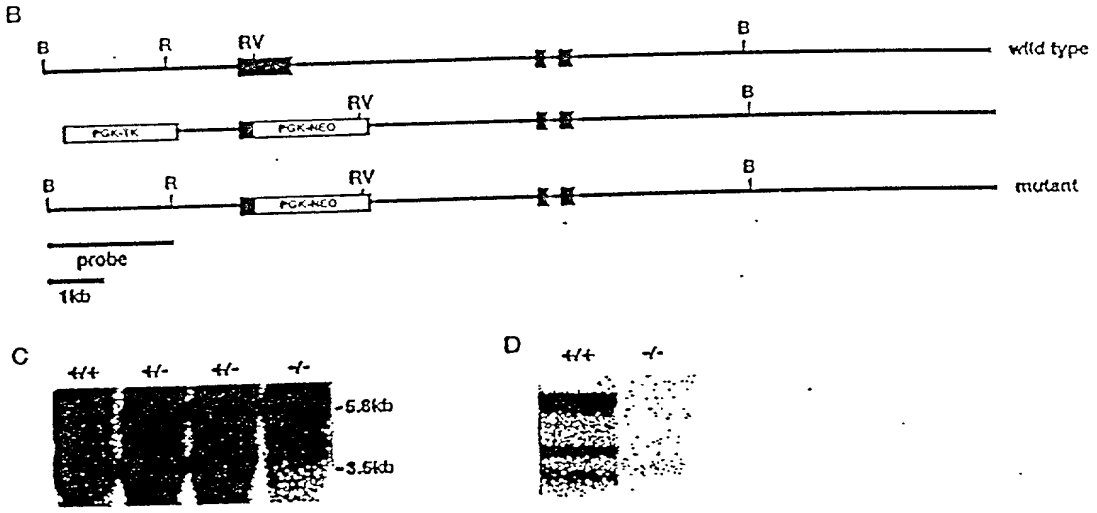


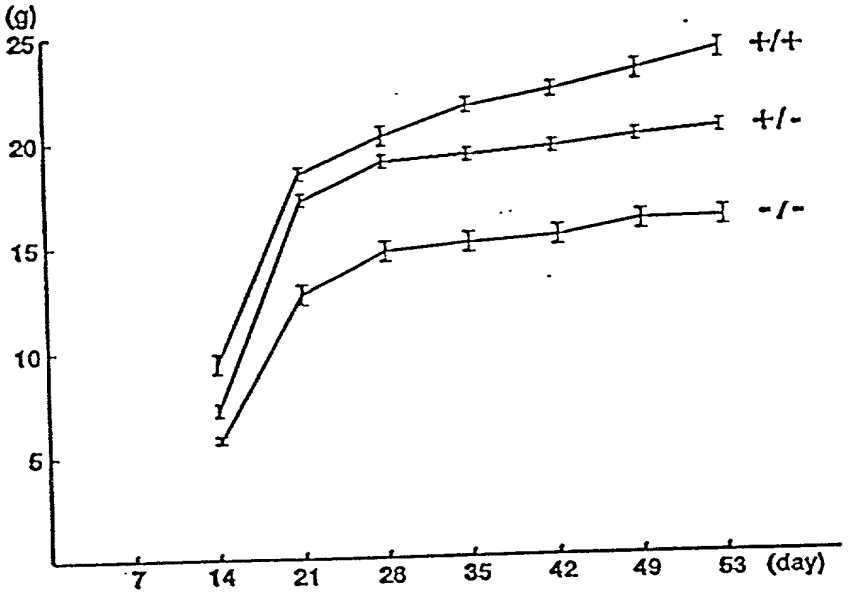
FIG. 6A


B
B
R
RV
B
wild type
RV
B
PGK-TK
PGK-NEO
B
R
RV
B
mutant
PGK-NEO
probe
1kb
C
+/+
+/-
+/-
-/-
6.8kb
3.5kb
D
+/+
-/-

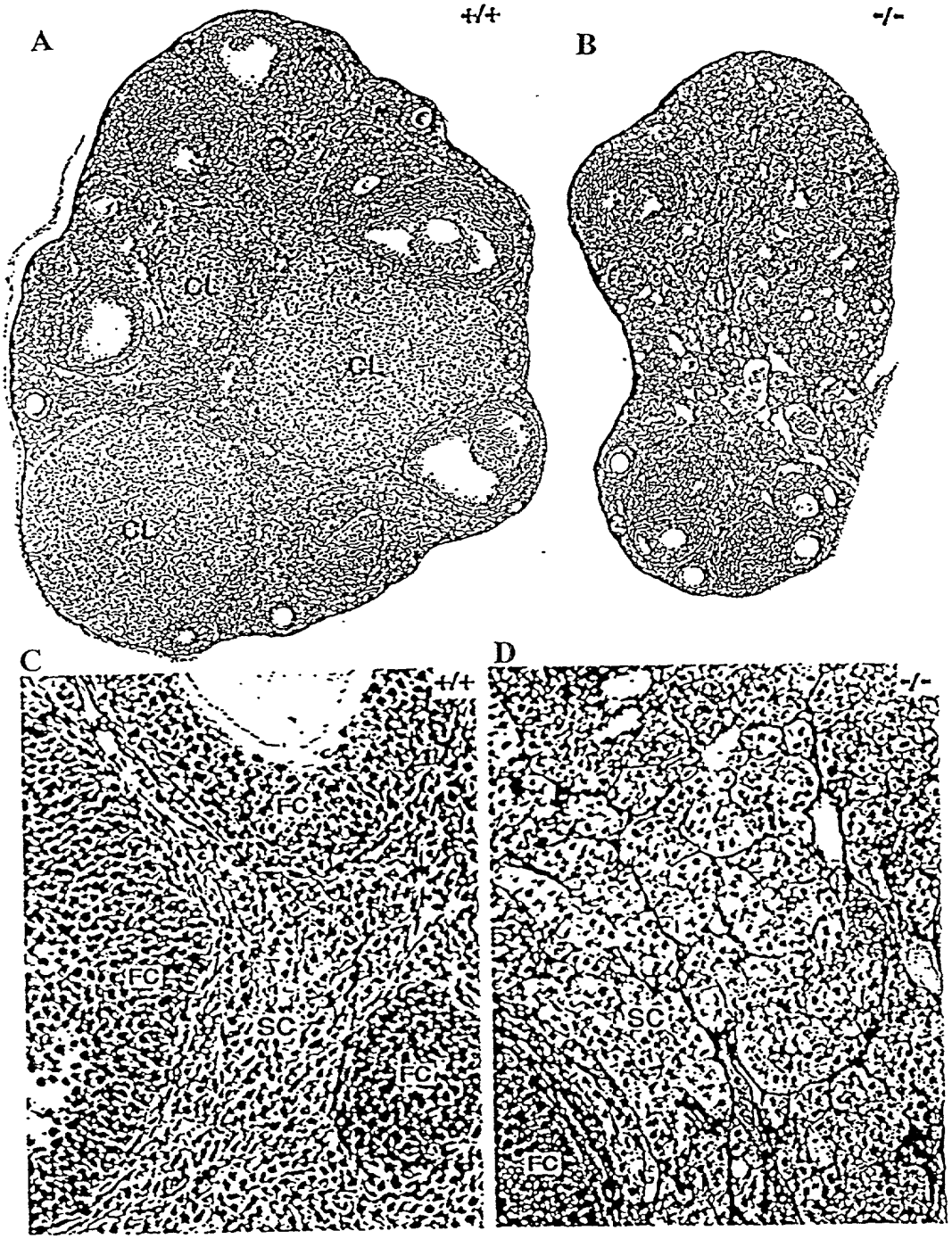
FIGS. 6B-D

A

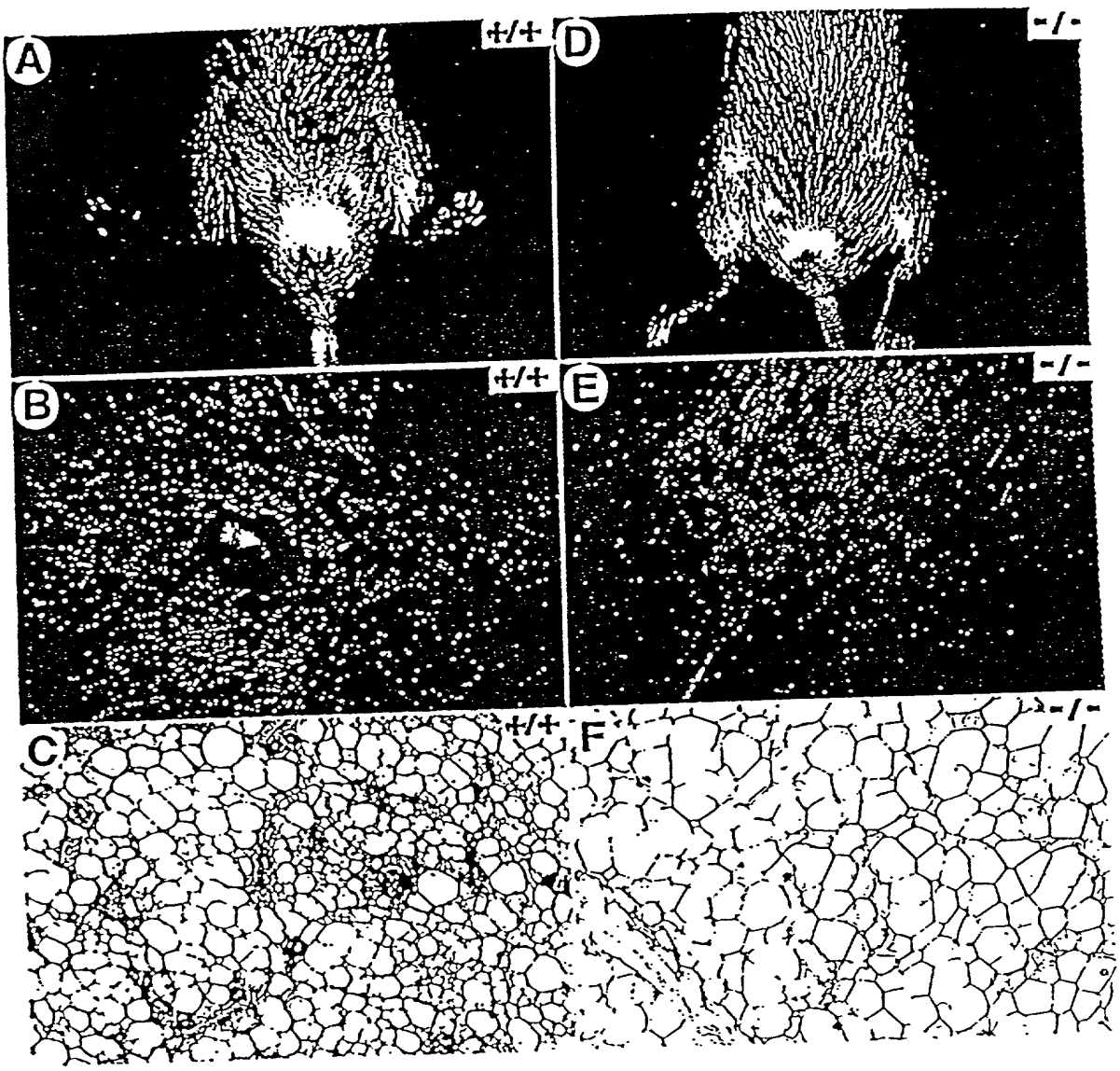
B



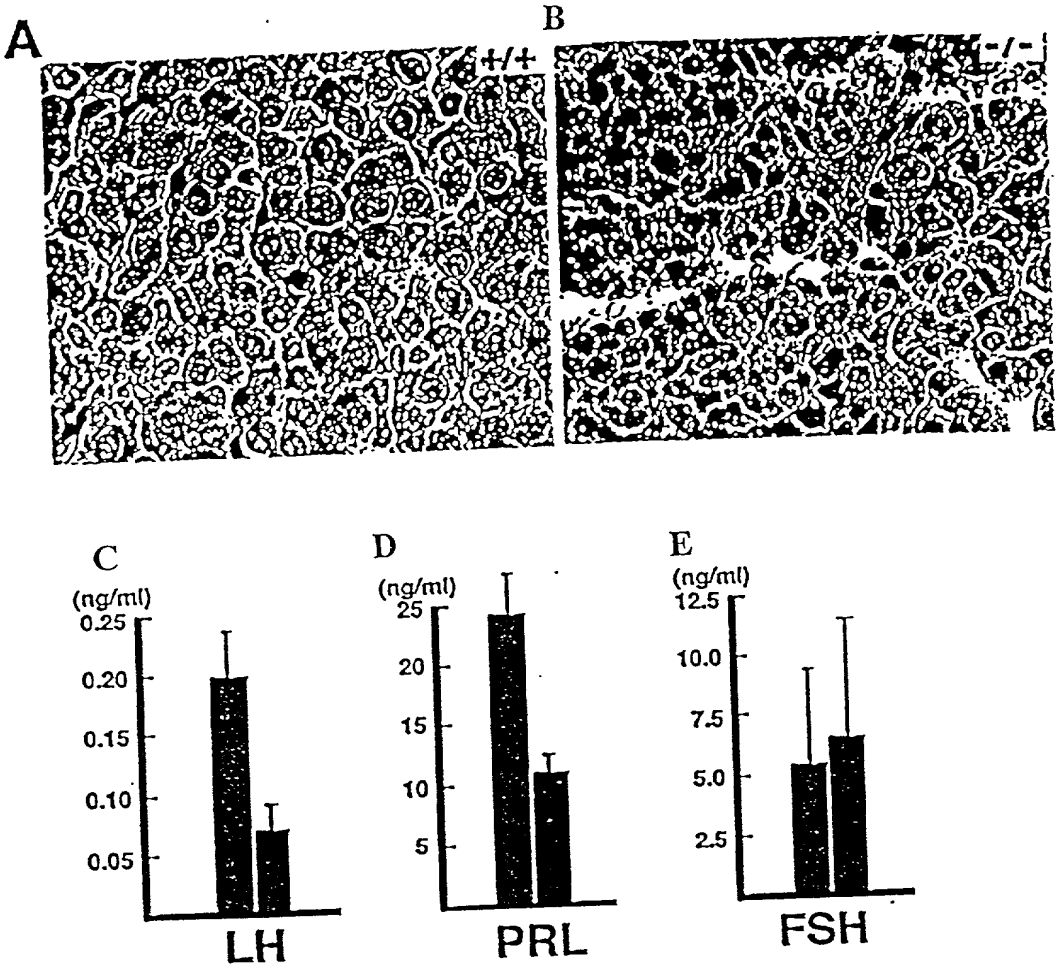
FIGS. 7A-B



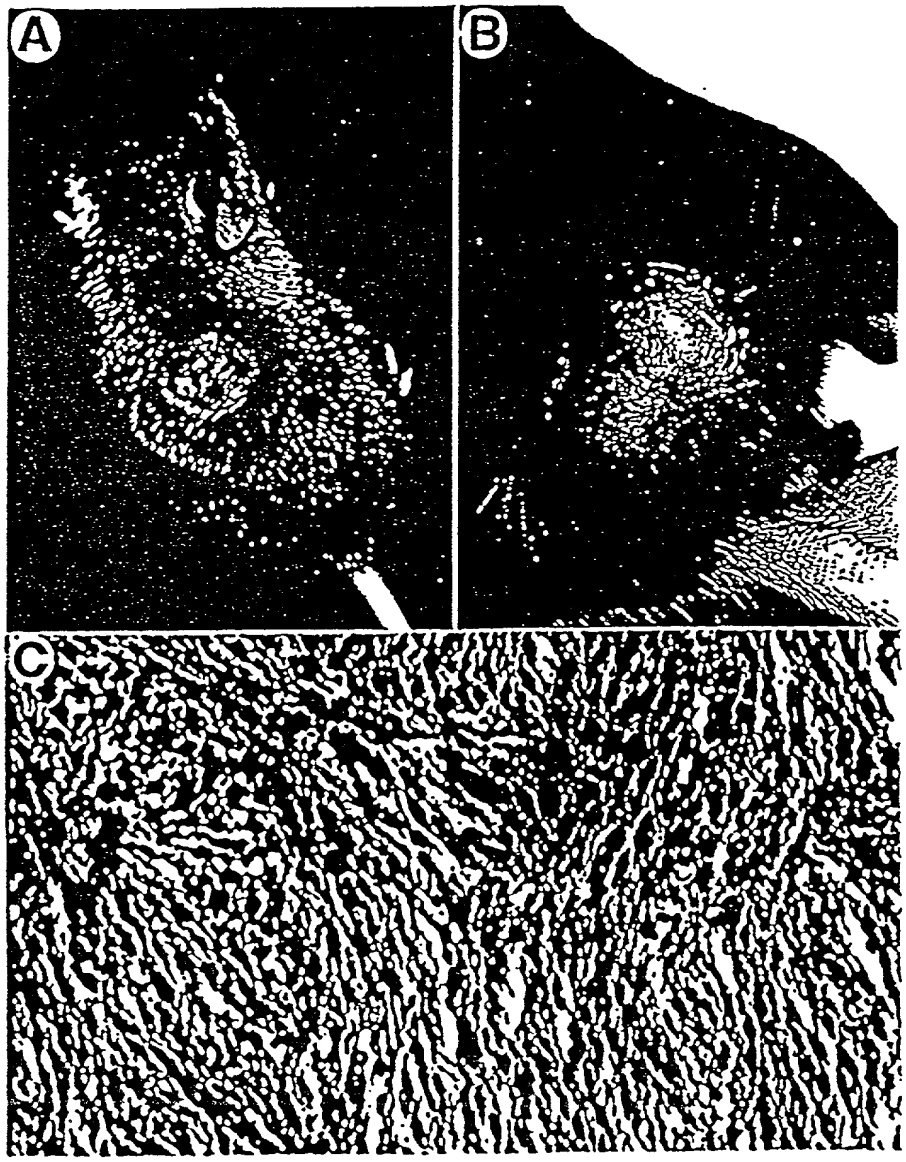
FIGS. 8A-D



FIGS. 9A-F


A
B
+/+
-/-
C
(ng/ml)
0.25
0.20
0.15
0.10
0.05
LH
D
(ng/ml)
25
20
15
10
5
PRL
E
(ng/ml)
12.5
10.0
7.5
5.0
2.5
FSH

FIGS. 10A-E



FIGS. 11A-C

```
        10         20         30         40         50         60         70         80
        *          *          *          *          *          *          *          *
ACCTTTGGGT TGCTGGGACG GACTCTGGCC GCCTCAGCGT CCGCCCTCAG GCCCGTGGCC GCTGTCCAGG AGCTCTGCTC

        90         100        110        120        130        140        150        160
        *          *          *          *          *          *          *          *
TCCCCTCCAG AGTTAATTAT TTATATTGTA AAGAATTTTA ACAGTCCTGG GGACTTCCTT GAAGGATCAT TTTCACTTTT

        170        180        190        200        210        220        230        240
        *          *          *          *          *          *          *          *
GCTCAGAAGA AAGCTCTGGA TCTATCAAAT AAAGAAGTCC TTCGTGTGGG CTACATATAT AGATGTTTTC ATGAAGAGGA
                                                                              M  K  R

        250        260        270        280        290        300        310        320
        *          *          *          *          *          *          *          *
GTGAAAAGCC AGAAGGATAT AGACAAATGA GGCCTAAGAC CTTTCCTGCC AGTAACTATA CTGTCAGTAG CCGGCAAATG
S  E  K  P  E  G  Y  R  Q  M  R  P  K  T  F  P  A  S  N  Y  T  V  S  S  R  Q  M

        330        340        350        360        370        380        390        400
        *          *          *          *          *          *          *          *
TTACAAGAAA TTCGGGAATC CCTTAGGAAT TTATCTAAAC CATCTGATGC TGCTAAGGCT GAGCATAACA TGAGTAAAAT
L  Q  E  I  R  E  S  L  R  N  L  S  K  P  S  D  A  A  K  A  E  H  N  M  S  K  M

        410        420        430        440        450        460        470        480
        *          *          *          *          *          *          *          *
GTCAACCGAA GATCCTCGAC AAGTCAGAAA TCCACCCAAA TTTGGGACGC ATCATAAAGC CTTGCAGGAA ATTCGAAACT
S  T  E  D  P  R  Q  V  R  N  P  P  K  F  G  T  H  H  K  A  L  Q  E  I  R  N

        490        500        510        520        530        540        550        560
        *          *          *          *          *          *          *          *
CTCTGCTTCC ATTTGCAAAT GAAACAAATT CTTCTCGGAG TACTTCAGAA GTTAATCCAC AAATGCTTCA AGACTTGCAA
S  L  L  P  F  A  N  E  T  N  S  S  R  S  T  S  E  V  N  P  Q  M  L  Q  D  L  Q

        570        580        590        600        610        620        630        640
        *          *          *          *          *          *          *          *
GCTGCTGGAT TTGATGAGGA TATGGTTATA CAAGCTCTTC AGAAAACTAA CAACAGAAGT ATAGAAGCAG CAATTGAATT
A  A  G  F  D  E  D  M  V  I  Q  A  L  Q  K  T  N  N  R  S  I  E  A  A  I  E  F

        650        660        670        680        690        700        710        720
        *          *          *          *          *          *          *          *
CATTAGTAAA ATGAGTTACC AAGATCCTCG ACGAGAGCAG ATGGCTGCAG CAGCTGCCAG ACCTATTAAT GCCAGCATGA
I  S  K  M  S  Y  Q  D  P  R  R  E  Q  M  A  A  A  A  A  R  P  I  N  A  S  M

        730        740        750        760        770        780        790        800
        *          *          *          *          *          *          *          *
AACCAGGGAA TGTGCAGCAA TCAGTTAACC GCAAACAGAG CTGGAAAGGT TCTAAAGAAT CCTTAGTTCC TCAGAGGCAT
K  P  G  N  V  Q  Q  S  V  N  R  K  Q  S  W  K  G  S  K  E  S  L  V  P  Q  R  H

        810        820        830        840        850        860        870        880
        *          *          *          *          *          *          *          *
GGCCCGCCAC TAGGAGAAAG TGTGGCCTAT CATTCTGAGA GTCCCAACTC ACAGACAGAT GTAGGAAGAC CTTTGTCTGG
G  P  P  L  G  E  S  V  A  Y  H  S  E  S  P  N  S  Q  T  D  V  G  R  P  L  S  G

        890        900        910        920        930        940        950        960
        *          *          *          *          *          *          *          *
ATCTGGTATA TCAGCATTTG TTCAAGCTCA CCCTAGCAAC GGACAGAGAG TGAACCCCCC ACCACCACCT CAAGTAAGGA
S  G  I  S  A  F  V  Q  A  H  P  S  N  G  Q  R  V  N  P  P  P  P  P  P  Q  V  R

        970        980        990        1000       1010       1020       1030       1040
        *          *          *          *          *          *          *          *
GTGTTACTCC TCCACCACCT CCAAGAGGCC AGACTCCCCC TCCAAGAGGT ACAACTCCAC CTCCCCCTTC ATGGGAACCA
S  V  T  P  P  P  P  P  R  G  Q  T  P  P  P  R  G  T  T  P  P  P  P  S  W  E  P
```

FIG. 12

```
      1050       1060       1070       1080       1090       1100       1110       1120
        *          *          *          *          *          *          *          *
AACTCTCAAA CAAAGCGCTA TTCTGGAAAC ATGGAATACG TAATCTCCCG AATCTCTCCT GTCCCACCTG GGGCATGGCA
 N  S  Q  T  K  R  Y  S  G  N  M  E  Y  V  I  S  R  I  S  P  V  P  P  G  A  W  Q

      1130       1140       1150       1160       1170       1180       1190       1200
        *          *          *          *          *          *          *          *
AGAGGGCTAT CCTCCACCAC CTCTCAACAC TTCCCCCATG AATCCTCCTA ATCAAGGACA GAGAGGCATT AGTTCTGTTC
 E  G  Y  P  P  P  P  L  N  T  S  P  M  N  P  P  N  Q  G  Q  R  G  I  S  S  V

      1210       1220       1230       1240       1250       1260       1270       1280
        *          *          *          *          *          *          *          *
CTGTTGGCAG ACAACCAATC ATCATGCAGA GTTCTAGCAA ATTTAACTTT CCATCAGGGA GACCTGGAAT GCAGAATGGT
 P  V  G  R  Q  P  I  I  M  Q  S  S  S  K  F  N  F  P  S  G  R  P  G  M  Q  N  G

      1290       1300       1310       1320       1330       1340       1350       1360
        *          *          *          *          *          *          *          *
ACTGGACAAA CTGATTTCAT GATACACCAA AATGTTGTCC CTGCTGGCAC TGTGAATCGG CAGCCACCAC CTCCATATCC
 T  G  Q  T  D  F  M  I  H  Q  N  V  V  P  A  G  T  V  N  R  Q  P  P  P  P  Y  P

      1370       1380       1390       1400       1410       1420       1430       1440
        *          *          *          *          *          *          *          *
TCTGACAGCA GCTAATGGAC AAAGCCCTTC TGCTTTACAA ACAGGGGGAT CTGCTGCTCC TTCGTCATAT ACAAATGGAA
 L  T  A  A  N  G  Q  S  P  S  A  L  Q  T  G  G  S  A  A  P  S  S  Y  T  N  G

      1450       1460       1470       1480       1490       1500       1510       1520
        *          *          *          *          *          *          *          *
GTATTCCTCA GTCTATGATG GTGCCAAACA GAAATAGTCA TAACATGGAA CTATATAACA TTAGTGTACC TGGACTGCAA
 S  I  P  Q  S  M  M  V  P  N  R  N  S  H  N  M  E  L  Y  N  I  S  V  P  G  L  Q

      1530       1540       1550       1560       1570       1580       1590       1600
        *          *          *          *          *          *          *          *
ACAAATTGGC CTCAGTCATC TTCTGCTCCA GCCCAGTCAT CCCCGAGCAG TGGGCATGAA ATCCCTACAT GGCAACCTAA
 T  N  W  P  Q  S  S  S  A  P  A  Q  S  S  P  S  S  G  H  E  I  P  T  W  Q  P  N

      1610       1620       1630       1640       1650       1660       1670       1680
        *          *          *          *          *          *          *          *
CATACCAGTG AGGTCAAATT CTTTTAATAA CCCATTAGGA AATAGAGCAA GTCACTCTGC TAATTCTCAG CCTTCTGCTA
 I  P  V  R  S  N  S  F  N  N  P  L  G  N  R  A  S  H  S  A  N  S  Q  P  S  A

      1690       1700       1710       1720       1730       1740       1750       1760
        *          *          *          *          *          *          *          *
CAACAGTCAC TGCAATTACA CCAGCTCCTA TTCAACAGCC TGTGAAAAGT ATGCGTGTAT TAAAACCAGA GCTACAGACT
 T  T  V  T  A  I  T  P  A  P  I  Q  Q  P  V  K  S  M  R  V  L  K  P  E  L  Q  T

      1770       1780       1790       1800       1810       1820       1830       1840
        *          *          *          *          *          *          *          *
GCTTTAGCAC CTACACACCC TTCTTGGATA CCACAGCCAA TTCAAACTGT TCAACCCAGT CCTTTTCCTG AGGGAACCGC
 A  L  A  P  T  H  P  S  W  I  P  Q  P  I  Q  T  V  Q  P  S  P  F  P  E  G  T  A

      1850       1860       1870       1880       1890       1900       1910       1920
        *          *          *          *          *          *          *          *
TTCAAATGTG ACTGTGATGC CACCTGTTGC TGAAGCTCCA AACTATCAAG GACCACCACC ACCCTACCCA AAACATCTGC
 S  N  V  T  V  M  P  P  V  A  E  A  P  N  Y  Q  G  P  P  P  P  Y  P  K  H  L

      1930       1940       1950       1960       1970       1980       1990       2000
        *          *          *          *          *          *          *          *
TGCACCAAAA CCCATCTGTT CCTCCATACG AGTCAATCAG TAAGCCTAGC AAAGAGGATC AGCCAAGCTT GCCCAAGGAA
 L  H  Q  N  P  S  V  P  P  Y  E  S  I  S  K  P  S  K  E  D  Q  P  S  L  P  K  E

      2010       2020       2030       2040       2050       2060       2070       2080
        *          *          *          *          *          *          *          *
GATGAGAGTG AAAAGAGTTA TGAAAATGTT GATAGTGGGG ATAAAGAAAA GAAACAGATT ACAACTTCAC CTATTACTGT
 D  E  S  E  K  S  Y  E  N  V  D  S  G  D  K  E  K  K  Q  I  T  T  S  P  I  T  V
```

FIG. 12 (cont.)

```
      2090       2100       2110       2120       2130       2140       2150       2160
       *          *          *          *          *          *          *          *
TAGGAAAAAC AAGAAAGATG AAGAGCGAAG GGAATCTCGT ATTCAAAGTT ATTCTCCTCA AGCATTTAAA TTCTTTATGG
  R  K  N    K  K  D    E  E  R  R    E  S  R    I  Q  S    Y  S  P  Q    A  F  K    F  F  M

      2170       2180       2190       2200       2210       2220       2230       2240
       *          *          *          *          *          *          *          *
AGCAACATGT AGAAAATGTA CTCAAATCTC ATCAGCAGCG TCTACATCGT AAAAAACAAT TAGAGAATGA AATGATGCGG
  E  Q  H  V    E  N  V    L  K  S    H  Q  Q  R    L  H  R    K  K  Q    L  E  N  E    M  M  R

      2250       2260       2270       2280       2290       2300       2310       2320
       *          *          *          *          *          *          *          *
GTTGGATTAT CTCAAGATGC CCAGGATCAA ATGAGAAAGA TGCTTTGCCA AAAAGAATCT AATTACATCC GTCTTAAAAG
  V  G  L    S  Q  D  A    Q  D  Q    M  R  K    M  L  C  Q    K  E  S    N  Y  I    R  L  K  R

      2330       2340       2350       2360       2370       2380       2390       2400
       *          *          *          *          *          *          *          *
GGCTAAAATG GACAAGTCTA TGTTTGTGAA GATAAAGACA CTAGGAATAG GAGCATTTGG TGAAGTCTGT CTAGCAAGAA
  A  K  M    D  K  S    M  F  V  K    I  K  T    L  G  I    G  A  F  G    E  V  C    L  A  R

      2410       2420       2430       2440       2450       2460       2470       2480
       *          *          *          *          *          *          *          *
AAGTAGATAC TAAGGCTTTG TATGCAACAA AAACTCTTCG AAAGAAAGAT GTTCTTCTTC GAAATCAAGT CGCTCATGTT
  K  V  D  T    K  A  L    Y  A  T  K    T  L  R    K  K  D    V  L  L    R  N  Q  V    A  H  V

      2490       2500       2510       2520       2530       2540       2550       2560
       *          *          *          *          *          *          *          *
AAGGCTGAGA GAGATATCCT GGCTGAAGCT GACAATGAAT GGGTAGTTCG TCTATATTAT TCATTCCAAG ATAAGGACAA
  K  A  E    R  D  I  L    A  E  A    D  N  E    W  V  V  R    L  Y  Y    S  F  Q    D  K  D  N

      2570       2580       2590       2600       2610       2620       2630       2640
       *          *          *          *          *          *          *          *
TTTATACTTT GTAATGGACT ACATTCCTGG GGGTGATATG ATGAGCCTAT TAATTAGAAT GGGCATCTTT CCAGAAAGTC
  L  Y  F    V  M  D    Y  I  P  G    G  D  M    M  S  L    L  I  R  M    G  I  F    P  E  S

      2650       2660       2670       2680       2690       2700       2710       2720
       *          *          *          *          *          *          *          *
TGGCACGATT CTACATAGCA GAACTTACCT GTGCAGTTGA AAGTGTTCAT AAAATGGGTT TTATTCATAG AGATATTAAA
  L  A  R  F    Y  I  A    E  L  T    C  A  V  E    S  V  H    K  M  G    F  I  H  R    D  I  K

      2730       2740       2750       2760       2770       2780       2790       2800
       *          *          *          *          *          *          *          *
CCTGATAATA TTTTGATTGA TCGTGATGGT CATATTAAAT TGACTGACTT TGGCCTCTGC ACTGGCTTCA GATGGACACA
  P  D  N    I  L  I  D    R  D  G    H  I  K    L  T  D  F    G  L  C    T  G  F    R  W  T  H

      2810       2820       2830       2840       2850       2860       2870       2880
       *          *          *          *          *          *          *          *
CGATTCTAAG TACTATCAGA GTGGTGACCA TCCACGGCAA GATAGCATGG ATTTCAGTAA TGAATGGGGG GATCCCTCAA
  D  S  K    Y  Y  Q    S  G  D  H    P  R  Q    D  S  M    D  F  S  N    E  W  G    D  P  S

      2890       2900       2910       2920       2930       2940       2950       2960
       *          *          *          *          *          *          *          *
GCTGTCGATG TGGAGACAGA CTGAAGCCAT TAGAGCGGAG AGCTGCACGC CAGCACCAGC GATGTCTAGC ACATTCTTTG
  S  C  R  C    G  D  R    L  K  P    L  E  R  R    A  A  R    Q  H  Q    R  C  L  A    H  S  L

      2970       2980       2990       3000       3010       3020       3030       3040
       *          *          *          *          *          *          *          *
GTTGGGACTC CCAATTATAT TGCACCTGAA GTGTTGCTAC GAACAGGATA CACACAGTTG TGTGATTGGT GGAGTGTTGG
  V  G  T    P  N  Y  I    A  P  E    V  L  L    R  T  G  Y    T  Q  L    C  D  W    W  S  V  G

      3050       3060       3070       3080       3090       3100       3110       3120
       *          *          *          *          *          *          *          *
TGTTATTCTT TTTGAAATGT TGGTGGGACA ACCTCCTTTC TTGGCACAAA CACCATTAGA AACACAAATG AAGGTTATCA
  V  I  L    F  E  M    L  V  G  Q    P  P  F    L  A  Q    T  P  L  E    T  Q  M    K  V  I
```

FIG. 12 (cont.)

```
      3130        3140        3150        3160        3170        3180        3190        3200
       *           *           *           *           *           *           *           *
ACTGGCAAAC ATCTCTTCAC ATTCCACCAC AAGCTAAACT CAGTCCTGAA GCTTCTGATC TTATTATTAA ACTTTGCCGA
N  W  Q  T   S  L  H   I  P  P   Q  A  K  L   S  P  E   A  S  D   L  I  I  K   L  C  R

      3210        3220        3230        3240        3250        3260        3270        3280
       *           *           *           *           *           *           *           *
GGACCCGAAG ATCGCTTAGG CAAGAATGGT GCTGATGAAA TAAAAGCTCA TCCATTTTTT AAAACAATTG ACTTCTCCAG
G  P  E   D  R  L  G   K  N  G   A  D  E   I  K  A  H   P  F  F   K  T  I   D  F  S  S

      3290        3300        3310        3320        3330        3340        3350        3360
       *           *           *           *           *           *           *           *
TGACCTGAGA CAGCAGTCTG CTTCATACAT TCCTAAAATC ACACACCCAA CAGATACATC AAATTTTGAT CCTGTTGATC
D  L  R   Q  Q  S   A  S  Y  I   P  K  I   T  H  P   T  D  T  S   N  F  D   P  V  D

      3370        3380        3390        3400        3410        3420        3430        3440
       *           *           *           *           *           *           *           *
CTGATAAATT ATGGAGTGAT GATAACGAGG AAGAAAATGT AAATGACACT CTCAATGGAT GGTATAAAAA TGGAAAGCAT
P  D  K  L   W  S  D   D  N  E   E  E  N  V   N  D  T   L  N  G   W  Y  K  N   G  K  H

      3450        3460        3470        3480        3490        3500        3510        3520
       *           *           *           *           *           *           *           *
CCTGAACATG CATTCTATGA ATTTACCTTC CGAAGGTTTT TTGATGACAA TGGCTACCCA TATAATTATC CGAAGCCTAT
P  E  H   A  F  Y  E   F  T  F   R  R  F   F  D  D  N   G  Y  P   Y  N  Y   P  K  P  I

      3530        3540        3550        3560        3570        3580        3590        3600
       *           *           *           *           *           *           *           *
TGAATATGAA TACATTAATT CACAAGGCTC AGAGCAGCAG TCGGATGAAG ATGATCAAAA CACAGGCTCA GAGATTAAAA
E  Y  E   Y  I  N   S  Q  G  S   E  Q  Q   S  D  E   D  D  Q  N   T  G  S   E  I  K

      3610        3620        3630        3640        3650        3660        3670        3680
       *           *           *           *           *           *           *           *
ATCGCGATCT AGTATATGTT TAACACACTA GTAAATAAAT GTAATGAGGA TTTGTAAAAG GGCCTGAAAT GCGAGGTGTT
N  R  D  L   V  Y  V   *

      3690        3700        3710        3720        3730        3740        3750        3760
       *           *           *           *           *           *           *           *
TTGAGGTTCT GAGAGTAAAA TTATGCAAAT ATGACAGAGC TATATATGTG TGCTCTGTGT ACAATATTTT ATTTTCCTAA

      3770        3780        3790        3800        3810        3820        3830        3840
       *           *           *           *           *           *           *           *
ATTATGGGAA ATCCTTTTAA AATGTTAATT TATTCCAGCC GTTTAAATCA GTATTTAGAA AAAAATTGTT ATAAGGAAAG

      3850        3860        3870        3880        3890        3900        3910        3920
       *           *           *           *           *           *           *           *
TAAATTATGA ACTGAATATT ATAGTCAGTT CTTGGTACTT AAAGTACTTA AAATAAGTAG TGCTTTGTTT AAAAGGAGAA

      3930        3940        3950        3960        3970        3980
       *           *           *           *           *           *
ACCTGGTATC TATTTGTATA TATGCTAAAT AATTTTAAAA TACAAGAGTT TTTGAAATTT TTTT
```

FIG. 12 (cont.)

```
        10         20         30         40         50         60         70         80
        *          *          *          *          *          *          *          *
GTGCAACATT CAATTAACCG AAAACAAAGC TGGAAAGGTT CTAAAGAGTC TCTAGTTCCT CAGAGACACG GCCCATCTCT
 V  Q  H    S  I  N  R    K  Q  S    W  K  G    S  K  E  S    L  V  P    Q  R  H    G  P  S  L

        90        100        110        120        130        140        150        160
        *          *          *          *          *          *          *          *
AGGAGAAAAT GTGGTTTATC GTTCTGAAAG CCCCAACTCA CAGGCGGATG TAGGAAGACC TCTGTCTGGA TCCGGCATTG
 G  E  N    V  V  Y    R  S  E  S    P  N  S    Q  A  D    V  G  R  P    L  S  G    S  G  I

       170        180        190        200        210        220        230        240
        *          *          *          *          *          *          *          *
CAGCATTTGC TCAAGCTCAC CCAAGCAATG GACAGAGAGT GAACCCCCCA CCACCACCTC AAGTTAGGAG TGTTACTCCT
 A  A  F  A    Q  A  H    P  S  N    G  Q  R  V    N  P  P    P  P  P    Q  V  R  S    V  T  P

       250        260        270        280        290        300        310        320
        *          *          *          *          *          *          *          *
CCACCACCTC CGAGAGGCCA GACCCCACCT CCCCGAGGCA CCACTCCCCC TCCCCCCTCA TGGGAACCAA GCTCTCAGAC
 P  P  P    P  R  G  Q    T  P  P    P  R  G    T  T  P  P    P  P  S    W  E  P    S  S  Q  T

       330        340        350        360        370        380        390        400
        *          *          *          *          *          *          *          *
AAAGCGCTAC TCTGGGAACA TGGAGTACGT AATCTCCCGA ATCTCCCCTG TTCCACCTGG GGCGTGGCAG GAGGGGTACC
 K  R  Y    S  G  N    M  E  Y  V    I  S  R    I  S  P    V  P  P  G    A  W  Q    E  G  Y

       410        420        430        440        450        460        470        480
        *          *          *          *          *          *          *          *
CTCCACCACC TCTTACCACT TCTCCCATGA ATCCCCCTAG CCAGGCTCAG AGGGCCATTA GTTCTGTTCC AGTTGGTAGA
 P  P  P  P    L  T  T    S  P  M    N  P  P  S    Q  A  Q    R  A  I    S  S  V  P    V  G  R

       490        500        510        520        530        540        550        560
        *          *          *          *          *          *          *          *
CAACCCATCA TCATGCAGAG TACTAGCAAA TTTAACTTTA CACCAGGGCG ACCTGGAGTT CAGAATGGTG GTGGTCAGTC
 Q  P  I    I  M  Q  S    T  S  K    F  N  F    T  P  G  R    P  G  V    Q  N  G    G  G  Q  S

       570        580        590        600        610        620        630        640
        *          *          *          *          *          *          *          *
TGATTTTATC GTGCACCAAA ATGTCCCCAC TGGTTCTGTG ACTCGGCAGC CACCACCTCC ATATCCTCTG ACCCCAGCTA
 D  F  I    V  H  Q    N  V  P  T    G  S  V    T  R  Q    P  P  P  P    Y  P  L    T  P  A

       650        660        670        680        690        700        710        720
        *          *          *          *          *          *          *          *
ATGGACAAAG CCCCTCTGCT TTACAAACAG GGGCTTCTGC TGCTCCACCA TCATTCGCCA ATGGAAACGT TCCTCAGTCG
 N  G  Q  S    P  S  A    L  Q  T    G  A  S  A    A  P  P    S  F  A    N  G  N  V    P  Q  S

       730        740        750        760        770        780        790        800
        *          *          *          *          *          *          *          *
ATGATGGTGC CCAACAGGAA CAGTCATAAC ATGGAGCTTT ATAATATTAA TGTCCCTGGA CTGCAAACAG CCTGGCCCCA
 M  M  V    P  N  R  N    S  H  N    M  E  L    Y  N  I  N    V  P  G    L  Q  T    A  W  P  Q

       810        820        830        840        850        860        870        880
        *          *          *          *          *          *          *          *
GTCGTCTTCT GCTCCTGCGC AGTCATCCCC AAGCGGTGGG CATGAAATTC CTACATGGCA ACCTAACATA CCAGTGAGGT
 S  S  S    A  P  A    Q  S  S  P    S  G  G    H  E  I    P  T  W  Q    P  N  I    P  V  R

       890        900        910        920        930        940        950        960
        *          *          *          *          *          *          *          *
CAAATTCTTT TAATAACCCA TTAGGAAGTA GAGCAAGTCA CTCTGCTAAT TCTCAGCCTT CTGCCACTAC AGTCACTGCC
 S  N  S  F    N  N  P    L  G  S    R  A  S  H    S  A  N    S  Q  P    S  A  T  T    V  T  A
```

FIG. 13

```
       970        980        990       1000       1010       1020       1030       1040
        *          *          *          *          *          *          *          *
ATCACACCCG CTCCTATTCA ACAGCCCGTG AAAAGCATGC GCGTCCTGAA ACCAGAGCTG CAGACTGCTT TAGCCCCAAC
 I  T  P    A  P  I  Q    Q  P  V    K  S  M    R  V  L  K    P  E  L    Q  T  A    L  A  P  T

      1050       1060       1070       1080       1090       1100       1110       1120
        *          *          *          *          *          *          *          *
CCATCCTTCT TGGATGCCAC AGCCAGTTCA GACTGTTCAG CCTACCCCTT TTTCTGAGGG TACAGCTTCA AGTGTGCCTG
 H  P  S    W  M  P    Q  P  V  Q    T  V  Q    P  T  P    F  S  E  G    T  A  S    S  V  P

      1130       1140       1150       1160       1170       1180       1190       1200
        *          *          *          *          *          *          *          *
TCATCCCACC TGTTGCTGAA GCTCCAAGCT ATCAAGGTCC ACCACCGCCT TATCCAAAAC ATCTGCTACA CCAAAACCCA
 V  I  P  P    V  A  E    A  P  S    Y  Q  G  P    P  P  P    Y  P  K    H  L  L  H    Q  N  P

      1210       1220       1230       1240       1250       1260       1270       1280
        *          *          *          *          *          *          *          *
TCTGTCCCTC CATATGAGTC AGTAAGTAAG CCCTGCAAAG ATGAACAGCC TAGCTTACCC AAGGAAGATG ATAGTGAGAA
 S  V  P    P  Y  E  S    V  S  K    P  C  K    D  E  Q  P    S  L  P    K  E  D    D  S  E  K

      1290       1300       1310       1320       1330       1340       1350       1360
        *          *          *          *          *          *          *          *
GAGTGCGGAC AGTGGTGACT CTGGGGATAA AGAAAAGAAA CAGATTACAA CTTCACCTAT CACTGTTCGG AAAAACAAGA
 S  A  D    S  G  D    S  G  D  K    E  K  K    Q  I  T    T  S  P  I    T  V  R    K  N  K

      1370       1380       1390       1400       1410       1420       1430       1440
        *          *          *          *          *          *          *          *
AAGATGAAGA ACGAAGAGAG TCTCGGATTC AGAGTTACTC CCCACAGGCC TTTAAGTTCT TCATGGAGCA GCACGTAGAG
K  D  E  E    R  R  E    S  R  I    Q  S  Y  S    P  Q  A    F  K  F    F  M  E  Q    H  V  E

      1450       1460       1470       1480       1490       1500       1510       1520
        *          *          *          *          *          *          *          *
AACGTCCTGA AGTCTCATCA GCAGCGTCTG CATCGGAAGA AGCAGCTAGA AAATGAAATG ATGCGGGTTG GATTATCTCA
N  V  L    K  S  H  Q    Q  R  L    H  R  K    K  Q  L  E    N  E  M    M  R  V    G  L  S  Q

      1530       1540       1550       1560       1570       1580       1590       1600
        *          *          *          *          *          *          *          *
AGATGCCCAG GATCAAATGA GAAAGATGCT TTGCCAGAAA GAGTCTAACT ATATTCGTCT TAAAAGGGCT AAAATGGACA
 D  A  Q    D  Q  M    R  K  M  L    C  Q  K    E  S  N    Y  I  R  L    K  R  A    K  M  D

      1610       1620       1630       1640       1650       1660       1670       1680
        *          *          *          *          *          *          *          *
AGTCTATGTT TGTAAAGATA AAGACATTAG GAATAGGAGC GTTTGGTGAA GTCTGTCTAG CAAGAAAAGT CGATACTAAA
K  S  M  F    V  K  I    K  T  L    G  I  G  A    F  G  E    V  C  L    A  R  K  V    D  T  K

      1690       1700       1710       1720       1730       1740       1750       1760
        *          *          *          *          *          *          *          *
GCTTTGTATG CAACAAAGAC TCTTCGAAAG AAAGACGTTC TGCTCCGAAA TCAGGTGGCT CATGTGAAAG CGGAGAGGGA
A  L  Y    A  T  K  T    L  R  K    K  D  V    L  L  R  N    Q  V  A    H  V  K    A  E  R  D

      1770       1780       1790       1800       1810       1820       1830       1840
        *          *          *          *          *          *          *          *
TATCCTAGCA GAAGCCGACA ATGAGTGGGT GGTCCGCCTG TACTACTCTT TCCAGGACAA GGACAACTTG TACTTTGTGA
 I  L  A    E  A  D    N  E  W  V    V  R  L    Y  Y  S    F  Q  D  K    D  N  L    Y  F  V

      1850       1860       1870       1880       1890       1900       1910       1920
        *          *          *          *          *          *          *          *
TGGACTACAT TCCTGGGGGG GATATGATGA GCCTATTAAT TAGAATGGGC ATCTTTCCTG AAAATCTGGC ACGATTCTAC
M  D  Y  I    P  G  G    D  M  M    S  L  L  I    R  M  G    I  F  P    E  N  L  A    R  F  Y

      1930       1940       1950       1960       1970       1980       1990       2000
        *          *          *          *          *          *          *          *
```

FIG. 13 (cont.)

```
ATAGCAGAAC TTACCTGTGC AGTTGAAAGT GTTCATAAAA TGGGTTTTAT TCATAGAGAT ATTAAACCTG ATAACATTTT
 I  A  E  L  T  C  A  V  E  S  V  H  K  M  G  F  I  H  R  D  I  K  P  D  N  I  L

      2010       2020       2030       2040       2050       2060       2070       2080
       *          *          *          *          *          *          *          *
GATTGACCGT GATGGCCATA TTAAATTGAC TGACTTTGGC TTGTGCACTG GCTTCAGATG GACACATGAC TCCAAGTACT
 I  D  R  D  G  H  I  K  L  T  D  F  G  L  C  T  G  F  R  W  T  H  D  S  K  Y

      2090       2100       2110       2120       2130       2140       2150       2160
       *          *          *          *          *          *          *          *
ACCAGAGTGG GGATCACCCA CGGCAAGATA GCATGGATTT CAGTAACGAA TGGGGAGATC CTTCCAATTG TCGGTGTGGG
 Y  Q  S  G  D  H  P  R  Q  D  S  M  D  F  S  N  E  W  G  D  P  S  N  C  R  C  G

      2170       2180       2190       2200       2210       2220       2230       2240
       *          *          *          *          *          *          *          *
GACAGACTGA AGCCACTGGA GCGGAGAGCT GCTCGCCAGC ACCAGCGATG TCTAGCCCAT TCTCTGGTTG GGACTCCCAA
 D  R  L  K  P  L  E  R  R  A  A  R  Q  H  Q  R  C  L  A  H  S  L  V  G  T  P  N

      2250       2260       2270       2280       2290       2300       2310       2320
       *          *          *          *          *          *          *          *
TTATATTGCA CCTGAAGTGC TACTGCGAAC AGGATATACA CAGCTGTGTG ACTGGTGGAG TGTTGGTGTT ATTCTTTGTG
 Y  I  A  P  E  V  L  L  R  T  G  Y  T  Q  L  C  D  W  W  S  V  G  V  I  L  C

      2330       2340       2350       2360       2370       2380       2390       2400
       *          *          *          *          *          *          *          *
AAATGTTGGT GGGACAACCT CCTTTCTTGG CACAAACCCC ATTAGAAACA CAAATGAAGG TTATCATCTG GCAAACTTCT
 E  M  L  V  G  Q  P  P  F  L  A  Q  T  P  L  E  T  Q  M  K  V  I  I  W  Q  T  S

      2410       2420       2430       2440       2450       2460       2470       2480
       *          *          *          *          *          *          *          *
CTACACATCC CTCCTCAAGC TAAGCTGAGT CCTGAAGCCT CTGACCTCAT TATCAAACTG TGTCGAGGAC CAGAAGACCG
 L  H  I  P  P  Q  A  K  L  S  P  E  A  S  D  L  I  I  K  L  C  R  G  P  E  D  R

      2490       2500       2510       2520       2530       2540       2550       2560
       *          *          *          *          *          *          *          *
CCTCGGCAAG AACGGTGCTG ATGAGATAAA GGCTCATCCA TTTTTTAAGA CCATCGATTT CTCTAGTGAT CTGAGACAGC
 L  G  K  N  G  A  D  E  I  K  A  H  P  F  F  K  T  I  D  F  S  S  D  L  R  Q

      2570       2580       2590       2600       2610       2620       2630       2640
       *          *          *          *          *          *          *          *
AGTCTGCTTC ATACATCCCT AAAATCACGC ATCCAACAGA TACATCCAAT TTCGACCCTG TTGATCCTGA TAAATTGTGG
 Q  S  A  S  Y  I  P  K  I  T  H  P  T  D  T  S  N  F  D  P  V  D  P  D  K  L  W

      2650       2660       2670       2680       2690       2700       2710       2720
       *          *          *          *          *          *          *          *
AGCGATGGCA GCGAGGAGGA AAATATCAGT GACACTCTGA GCGGATGGTA TAAAAATGGG AAGCACCCCG AGCACGCTTT
 S  D  G  S  E  E  E  N  I  S  D  T  L  S  G  W  Y  K  N  G  K  H  P  E  H  A  F

      2730       2740       2750       2760       2770       2780       2790       2800
       *          *          *          *          *          *          *          *
CTATGAGTTC ACCTTTCGGA GGTTTTTTGA TGACAATGGC TACCCATATA ATTATCCAAA GCCTATTGAG TATGAATACA
 Y  E  F  T  F  R  R  F  F  D  D  N  G  Y  P  Y  N  Y  P  K  P  I  E  Y  E  Y

      2810       2820       2830       2840       2850       2860       2870       2880
       *          *          *          *          *          *          *          *
TTCATTCACA GGGCTCAGAA CAACAGTCTG ATGAAGATGA TCAACACACA AGCTCCGATG GAAACAACCG AGATCTAGTG
 I  H  S  Q  G  S  E  Q  Q  S  D  E  D  D  Q  H  T  S  S  D  G  N  N  R  D  L  V

      2890       2900       2910       2920       2930       2940       2950       2960
       *          *          *          *          *          *          *          *
TATGTTTAAT AAACTAGGAG ATCATTGTAA GAATTTGCAA GAGGCCTGAA GTGCAGGGGT TTTTGAAGTT TTGAGAAAAT
 Y  V  *
```

FIG. 13 (cont.)

```
      2970       2980       2990       3000       3010       3020       3030       3040
       *          *          *          *          *          *          *          *
TATGCAAATG TGACAGAGTT TGTGTGCTCT GTGTACAATA TTTTATTTTC CTAAGTTATG GGAAATTGTT TTAAAATGTT

      3050       3060       3070       3080       3090       3100       3110       3120
       *          *          *          *          *          *          *          *
AATTTATTCC ACCCTTTTAA TTCAGTAATT TAGAAAAAAT TGTTATAAGG AAAGTAAATT ATGAACTGAG TATTATAGTC

      3130       3140       3150       3160       3170       3180       3190       3200
       *          *          *          *          *          *          *          *
AATTCTTGGT ACTTAAAGTA CTTAAAAAGA GAAGCCTGGT ATCTTTTGTA TATATAATAA ATAATTTTAA AATCCCAAAA

      3210
       *
AAAAAAAAAA AAA
```

FIG. 13 (cont.)

```
        10         20         30         40         50         60         70         80
         *          *          *          *          *          *          *          *
ATGAGAGCCA CCCCGAAGTT TGGACCTTAT CAAAAAGCTC TCAGGGAAAT CCGATATTCC CTCCTGCCTT TTGCCAACGA
 M  R  A  T  P  K  F  G  P  Y  Q  K  A  L  R  E  I  R  Y  S  L  L  P  F  A  N  E

        90        100        110        120        130        140        150        160
         *          *          *          *          *          *          *          *
GTCAGGCACT TCGGCAGCTG CAGAGGTGAA CCGGCAGATG CTTCAGGAGT TGGTGAATGC GGCATGTGAC CAGGAGATGG
 S  G  T  S  A  A  A  E  V  N  R  Q  M  L  Q  E  L  V  N  A  A  C  D  Q  E  M

       170        180        190        200        210        220        230        240
         *          *          *          *          *          *          *          *
CTGGCAGAGC GCTCACGCAG ACGGGCAGTA GGAGTATCGA AGCTGCCTTG GAGTACATCA GTAAGATGGG CTACCTGGAC
 A  G  R  A  L  T  Q  T  G  S  R  S  I  E  A  A  L  E  Y  I  S  K  M  G  Y  L  D

       250        260        270        280        290        300        310        320
         *          *          *          *          *          *          *          *
CCCAGGAATG AGCAGATTGT GCGAGTCATC AAGCAGACCT CCCCAGGAAA GGGCCTGGCG TCCACCCCGG TGACTCGGCG
 P  R  N  E  Q  I  V  R  V  I  K  Q  T  S  P  G  K  G  L  A  S  T  P  V  T  R  R

       330        340        350        360        370        380        390        400
         *          *          *          *          *          *          *          *
GCCCAGTTTC GAGGGCACAG GGGAAGCACT CCCATCCTAC CACCAGCTGG GTGGTGCAAA CTACGAGGGC CCCGCCGCAC
 P  S  F  E  G  T  G  E  A  L  P  S  Y  H  Q  L  G  G  A  N  Y  E  G  P  A  A

       410        420        430        440        450        460        470        480
         *          *          *          *          *          *          *          *
TGGAGGAGAT GCCGCGGCAA TATTTAGACT TCTCTTCCCC TGGAGCCGGA GCCGGCACCC ACGGTGCCCA GGCTCACCAG
 L  E  E  M  P  R  Q  Y  L  D  F  L  F  P  G  A  G  A  G  T  H  G  A  Q  A  H  Q

       490        500        510        520        530        540        550        560
         *          *          *          *          *          *          *          *
CATCCTCCCA AAGGGTACAG CACAGCAGTA GAGCCAAGTG CGCACTTTCC GGGCACACAC TATGGTCGTG GTCATCTACT
 H  P  P  K  G  Y  S  T  A  V  E  P  S  A  H  F  P  G  T  H  Y  G  R  G  H  L  L

       570        580        590        600        610        620        630        640
         *          *          *          *          *          *          *          *
ATCGGAGCAG TCTGGGTATG GGGTGCAGCG CAGTTCCTCC TTCCAGAACA AGACGCCACC AGATGCCTAT TCCAGCATGG
 S  E  Q  S  G  Y  G  V  Q  R  S  S  S  F  Q  N  K  T  P  P  D  A  Y  S  S  M

       650        660        670        680        690        700        710        720
         *          *          *          *          *          *          *          *
CCAAGGCCCA GGGTGGCCCT CCCGCCAGCC TCACCTTTCC TGCCCATGCT GGGCTGTACA CTGCCTCGCA CCACAAGCCG
 A  K  A  Q  G  G  P  P  A  S  L  T  F  P  A  H  A  G  L  Y  T  A  S  H  H  K  P

       730        740        750        760        770        780        790        800
         *          *          *          *          *          *          *          *
GCGGCTACCC CACCTGGGGC CCACCCATTA CATGTGTTGG GCACCCGGGG TCCCACGTTT ACTGGCGAAA GCTCTGCACA
 A  A  T  P  P  P  G  A  H  P  L  H  V  L  G  T  R  G  P  T  F  T  G  E  S  S  A  Q

       810        820        830        840        850        860        870        880
         *          *          *          *          *          *          *          *
GGCTGTGCTG GCACCGTCCA GGAACAGCCT CAATGCTGAC TTGTACGAGC TGGGCTCCAC GGTGCCCTGG TCTGCAGCTC
 A  V  L  A  P  S  R  N  S  L  N  A  D  L  Y  E  L  G  S  T  V  P  W  S  A  A

       890        900        910        920        930        940        950        960
         *          *          *          *          *          *          *          *
CACTGGCACG CCGCGACTCG CTGCAGAAGC AGGGTCTAGA AGCCTCGCGG CCGCATGTGG CTTTTCGGGC TGGCCCCAGC
 P  L  A  R  R  D  S  L  Q  K  Q  G  L  E  A  S  R  P  H  V  A  F  R  A  G  P  S
```

FIG. 14

```
      970        980        990       1000       1010       1020       1030       1040
       *          *          *          *          *          *          *          *
AGGACCAACT CCTTCAACAA CCCACAACCT GAGCCCTCAC TGCCCGCCCC CAACACGGTC ACCGCCGTGA CGGCCGCACA
 R  T  N    S  F  N  N    P  Q  P    E  P  S    L  P  A  P    N  T  V    T  A  V    T  A  A  H

      1050       1060       1070       1080       1090       1100       1110       1120
       *          *          *          *          *          *          *          *
CATCCTTCAC CCTGTGAAGA GCGTGCGTGT GCTGCGGCCC GAGCCCCAGA CAGCCGTGGG GCCCTCGCAC CCCGCCTGGG
 I  L  H    P  V  K    S  V  R  V    L  R  P    E  P  Q    T  A  V  G    P  S  H    P  A  W

      1130       1140       1150       1160       1170       1180       1190       1200
       *          *          *          *          *          *          *          *
TGGCTGCGCC CACAGCACCT GCCACTGAGA GCCTGGAGAC GAAGGAGGGC AGCGCAGGCC CACACCCGCT GGATGTGGAC
 V  A  A  P    T  A  P    A  T  E    S  L  E  T    K  E  G    S  A  G    P  H  P  L    D  V  D

      1210       1220       1230       1240       1250       1260       1270       1280
       *          *          *          *          *          *          *          *
TATGGCGGCT CCGAGCGCAG GTGCCCACCG CCTCCGTATC CAAAGCACTT GCTGCTGCCC AGTAAGTCTG AGCAGTACAG
 Y  G  G    S  E  R  R    C  P  P    P  P  Y    P  K  H  L    L  L  P    S  K  S    E  Q  Y  S

      1290       1300       1310       1320       1330       1340       1350       1360
       *          *          *          *          *          *          *          *
CGTGGACCTG GACAGCCTGT GCACCAGTGT GCAGCAGAGT CTGCGAGGGG GCACTGATCT AGACGGGAGT GACAAGAGCC
 V  D  L    D  S  L    C  T  S  V    Q  Q  S    L  R  G    G  T  D  L    D  G  S    D  K  S

      1370       1380       1390       1400       1410       1420       1430       1440
       *          *          *          *          *          *          *          *
ACAAAGGTGC GAAGGGAGAC AAAGCTGGCA GAGACAAAAA GCAGATTCAG ACCTCCCCGG TGCCTGTCCG CAAGAATAGC
 H  K  G    A  K  G    D  K  A  G    R  D  K    K  Q  I    Q  T  S    P  V  P  V    R  K  N  S

      1450       1460       1470       1480       1490       1500       1510       1520
       *          *          *          *          *          *          *          *
AGAGATGAAG AGAAGAGAGA GTCTCGCATC AAGAGTTACT CCCCTTATGC CTTCAAATTC TTCATGGAGC AACACGTGGA
 R  D  E    E  K  R  E    S  R  I    K  S  Y    S  P  Y  A    F  K  F    F  M  E    Q  H  V  E

      1530       1540       1550       1560       1570       1580       1590       1600
       *          *          *          *          *          *          *          *
GAATGTCATC AAAACCTACC AGCAGAAGGT CAGCCGGAGG CTACAGCTGG AGCAGGAAAT GGCCAAAGCT GGGCTCTGTG
 N  V  I    K  T  Y    Q  Q  K  V    S  R  R    L  Q  L    E  Q  E  M    A  K  A    G  L  C

      1610       1620       1630       1640       1650       1660       1670       1680
       *          *          *          *          *          *          *          *
AGGCCGAGCA GGAGCAGATG AGGAAGATCC TCTACCAGAA GGAGTCTAAC TACAACCGGC TGAAGAGGGC CAAGATGGAC
 E  A  E  Q    E  Q  M    R  K  I    L  Y  Q  K    E  S  N    Y  N  R    L  K  R  A    K  M  D

      1690       1700       1710       1720       1730       1740       1750       1760
       *          *          *          *          *          *          *          *
AAGTCCATGT TTGTGAAAAT CAAGACTCTA GGCATCGGTG CCTTTGGGGA AGTGTGCCTC GCTTGTAAGC TGGACACTCA
 K  S  M    F  V  K  I    K  T  L    G  I  G    A  F  G  E    V  C  L    A  C  K    L  D  T  H

      1770       1780       1790       1800       1810       1820       1830       1840
       *          *          *          *          *          *          *          *
CGCTCTGTAC GCCATGAAGA CTCTCAGGAA GAAGGATGTC CTGAACCGGA ATCAAGTGGC CCATGTCAAG GCTGAGAGGG
 A  L  Y    A  M  K    T  L  R  K    K  D  V    L  N  R    N  Q  V  A    H  V  K    A  E  R

      1850       1860       1870       1880       1890       1900       1910       1920
       *          *          *          *          *          *          *          *
ACATCCTGGC TGAAGCAGAC AATGAGTGGG TGGTCAAACT CTACTACTCC TTCCAGGACA AGGACAGCCT GTACTTTGTG
 D  I  L  A    E  A  D    N  E  W    V  V  K  L    Y  Y  S    F  Q  D  K    D  S  L    Y  F  V

      1930       1940       1950       1960       1970       1980       1990       2000
       *          *          *          *          *          *          *          *
```

FIG. 14 (cont.)

```
ATGGACTACA TACCAGGCGG GGATATGATG AGCCTGCTGA TCAGGATGGA GGTCTTCCCT GAGCACCTGG CCCGCTTCTA
 M  D  Y  I  P  G  G  D  M  M  S  L  L  I  R  M  E  V  F  P  E  H  L  A  R  F  Y

      2010       2020       2030       2040       2050       2060       2070       2080
       *          *          *          *          *          *          *          *
CATTGCAGAG TTGACCCTGG CCATTGAAAG TGTCCACAAG ATGGGCTTTA TCCACCGGGA CATCAAGCCT GACAACATAC
 I  A  E  L  T  L  A  I  E  S  V  H  K  M  G  F  I  H  R  D  I  K  P  D  N  I

      2090       2100       2110       2120       2130       2140       2150       2160
       *          *          *          *          *          *          *          *
TCATCGACCT GGATGGTCAT ATTAAGCTGA CAGATTTTGG CCTCTGCACT GGATTCAGGT GGACTCACAA TTCCAAGTAC
 L  I  D  L  D  G  H  I  K  L  T  D  F  G  L  C  T  G  F  R  W  T  H  N  S  K  Y

      2170       2180       2190       2200       2210       2220       2230       2240
       *          *          *          *          *          *          *          *
TACCAGAAAG GGAACCACAT GAGACAGGAC AGCATGGAGC CCGGTGACCT CTGGGACGAT GTTTCCAACT GTCGCTGTGG
 Y  Q  K  G  N  H  M  R  Q  D  S  M  E  P  G  D  L  W  D  D  V  S  N  C  R  C  G

      2250       2260       2270       2280       2290       2300       2310       2320
       *          *          *          *          *          *          *          *
AGACAGGTTA AAGACCCTGG AGCAGAGGGC GCAGAAGCAG CACCAGAGGT GCCTGGCACA TTCTCTTGTC GGGACACCAA
 D  R  L  K  T  L  E  Q  R  A  Q  K  Q  H  Q  R  C  L  A  H  S  L  V  G  T  P

      2330       2340       2350       2360       2370       2380       2390       2400
       *          *          *          *          *          *          *          *
ATTACATCGC TCCGGAGGTG CTTCTCCGCA AAGGGTACAC GCAGCTCTGT GACTGGTGGA GCGTCGGTGT GATTCTCTTT
 N  Y  I  A  P  E  V  L  L  R  K  G  Y  T  Q  L  C  D  W  W  S  V  G  V  I  L  F

      2410       2420       2430       2440       2450       2460       2470       2480
       *          *          *          *          *          *          *          *
GAGATGCTGG TTGGGCAGCC GCCTTTCTTG GCCCCCACCC CCACAGAGAC GCAGCTGAAG GTGATCAACT GGGAGAGCAC
 E  M  L  V  G  Q  P  P  F  L  A  P  T  P  T  E  T  Q  L  K  V  I  N  W  E  S  T

      2490       2500       2510       2520       2530       2540       2550       2560
       *          *          *          *          *          *          *          *
GCTGCATATC CCTACGCAGG TGAGGCTCAG CGCTGAGGCC CGAGACCTCA TCACGAAGCT GTGCTGCGCG GCTGACTGCC
 L  H  I  P  T  Q  V  R  L  S  A  E  A  R  D  L  I  T  K  L  C  C  A  A  D  C

      2570       2580       2590       2600       2610       2620       2630       2640
       *          *          *          *          *          *          *          *
GCCTGGGCAG GGATGGGGCA GATGACCTCA AGGCACACCC GTTCTTCAAC ACCATCGACT TTTCCCGTGA CATCCGAAAG
 R  L  G  R  D  G  A  D  D  L  K  A  H  P  F  F  N  T  I  D  F  S  R  D  I  R  K

      2650       2660       2670       2680       2690       2700       2710       2720
       *          *          *          *          *          *          *          *
CAGGCTGCAC CCTACGTCCC CACCATCAGC CACCCCATGG ACACCTCCAA TTTTGACCCG GTGGATGAAG AAAGCCCCTG
 Q  A  A  P  Y  V  P  T  I  S  H  P  M  D  T  S  N  F  D  P  V  D  E  E  S  P  W

      2730       2740       2750       2760       2770       2780       2790       2800
       *          *          *          *          *          *          *          *
GCACGAGGCC AGCGGAGAGA GCGCCAAGGC CTGGGACACG CTGGCCTCCC CCAGCAGCAA GCATCCAGAG CACGCCTTCT
 H  E  A  S  G  E  S  A  K  A  W  D  T  L  A  S  P  S  S  K  H  P  E  H  A  F

      2810       2820       2830       2840       2850       2860       2870       2880
       *          *          *          *          *          *          *          *
ATGAGTTCAC CTTCCGCAGG TTCTTCGATG ACAACGGCTA TCCCTTCCGG TGCCCGAAGC CCTCAGAGCC CGCAGAGAGT
 Y  E  F  T  F  R  R  F  F  D  D  N  G  Y  P  F  R  C  P  K  P  S  E  P  A  E  S

      2890       2900       2910       2920       2930       2940       2950       2960
       *          *          *          *          *          *          *          *
GCAGACCCAG GGGATGCGGA CTTGGAAGGT GCGGCCGAGG GCTGCCAGCC GGTGTACGTG TAAGCCTCAG TTAACCACAA
 A  D  P  G  D  A  D  L  E  G  A  A  E  G  C  Q  P  V  Y  V  *
```

FIG. 14 (cont.)

```
      2970        2980        2990        3000        3010        3020        3030        3040
        *           *           *           *           *           *           *           *
CTCGAGGAAA CCCAAAATGA GATTTCTTTT CAGAAGACAA ACTCAAGCTT AGGAATCCTT CATTTTTAGT TCTGGTAAAT

      3050        3060        3070        3080        3090        3100        3110        3120
        *           *           *           *           *           *           *           *
GGGCAACAGG AAGAGTCAAC ATGATTTCAA ATTAGCCCTC TGAGGACCTT CACTGCATTA AAACAGTATT TTTTAAAAAA

      3130        3140        3150
        *           *           *
TTAGTACAGT ATGGAAAGAG CACTTATTTT GGGGG
```

FIG. 14 (cont.)

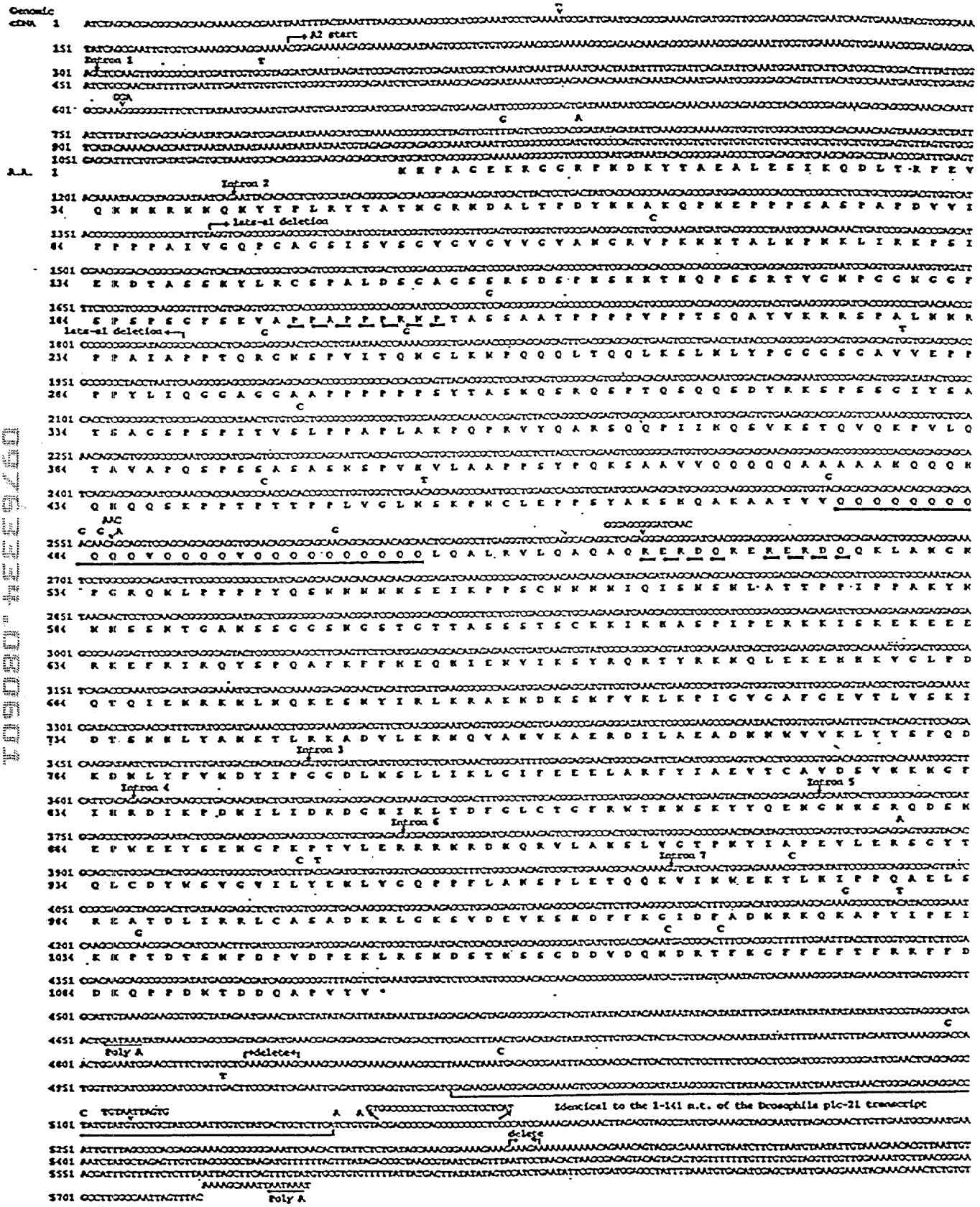

Genomic
cDNA
A2 start
Intron 1
Intron 2
late-el deletion
Intron 3
Intron 4
Intron 5
Intron 6
Intron 7
Poly A
delete
Identical to the 1-141 n.t. of the Drosophila plc-21 transcript

FIG. 15

DECLARATION FOR NON-PROVISIONAL PATENT APPLICATION*

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below at 201 et seq. beneath my name.

I believe I am the original, first and sole inventor if only one name is listed at 201 below, or an original, first and joint inventor if plural names are listed at 201 et seq. below, of the subject matter which is claimed and for which a patent is sought on the invention entitled

TREATMENT AND PREVENTION OF CANCER AND PITUITARY DISORDERS WITH LATS PROTEINS, DERIVATIVES AND FRAGMENTS, AND LATS KNOCK-OUT ANIMAL MODELS

and for which a patent application:
☐ is attached hereto and includes amendment(s) filed on *(if applicable)*
☒ was filed in the United States on February 16, 2001 as Application No. 09/763,334 *(for declaration not accompanying application)*
with amendment(s) filed on *(if applicable)*
☒ was filed as PCT international Application No. PCT/US99/19068 on August 18, 1999 and was amended under PCT Article 19 on *(if applicable)*

I hereby state that I have reviewed and understand the contents of the above identified application, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, §1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, §119(a)-(d) of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

EARLIEST FOREIGN APPLICATION(S), IF ANY, FILED PRIOR TO THE FILING DATE OF THE APPLICATION			
APPLICATION NUMBER	COUNTRY	DATE OF FILING (day, month, year)	PRIORITY CLAIMED
			YES ☐ NO ☐
			YES ☐ NO ☐
			YES ☐ NO ☐

I hereby claim the benefit under Title 35, United States Code, §119(e) of any United States provisional application(s) listed below.

PROVISIONAL APPLICATION NUMBER	FILING DATE
60/096,996	August 18, 1998
60,096,997	August 18, 1998

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code §112, I acknowledge the duty to disclose information known to me which is material to patentability as defined in Title 37, Code of Federal Regulations, §1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application:

NON-PROVISIONAL APPLICATION SERIAL NO.	FILING DATE	STATUS		
		PATENTED	PENDING	ABANDONED

* for use only when the application is assigned to a company, partnership or other organization.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

201	FULL NAME OF INVENTOR	LAST NAME: Xu	FIRST NAME: Tian	MIDDLE NAME:	
	RESIDENCE & CITIZENSHIP	CITY: Guilford	STATE OR FOREIGN COUNTRY: Connecticut	COUNTRY OF CITIZENSHIP: U.S.A.	
	POST OFFICE ADDRESS	STREET: 149 Flag Marsh Road	CITY: Guilford	STATE OR COUNTRY: CT	ZIP CODE: 06437
		SIGNATURE OF INVENTOR 201		DATE	
202	FULL NAME OF INVENTOR	LAST NAME: Tao	FIRST NAME: Wufan	MIDDLE NAME:	
	RESIDENCE & CITIZENSHIP	CITY: Minneapolis	STATE OR FOREIGN COUNTRY: Minnesota	COUNTRY OF CITIZENSHIP: U.S.A.	
	POST OFFICE ADDRESS	STREET: Box 806 Mayo 420 Delaware Street SE	CITY: Minneapolis	STATE OR COUNTRY: MN	ZIP CODE: 55455
		SIGNATURE OF INVENTOR 202		DATE	
203	FULL NAME OF INVENTOR	LAST NAME: St. John	FIRST NAME: Maie	MIDDLE NAME: A.R.	
	RESIDENCE & CITIZENSHIP	CITY: Los Angeles	STATE OR FOREIGN COUNTRY: California	COUNTRY OF CITIZENSHIP: U.S.A.	
	POST OFFICE ADDRESS	STREET: 3008 Motor Avenue	CITY: Los Angeles	STATE OR COUNTRY: CA	ZIP CODE: 90064
		SIGNATURE OF INVENTOR 203		DATE	
204	FULL NAME OF INVENTOR	LAST NAME: Fei	FIRST NAME: Xiaolan	MIDDLE NAME:	
	RESIDENCE & CITIZENSHIP	CITY: New Haven	STATE OR FOREIGN COUNTRY: Connecticut	COUNTRY OF CITIZENSHIP: U.S.A.	
	POST OFFICE ADDRESS	STREET: 393 Mansfield Street, Apt. 21	CITY: New Haven	STATE OR COUNTRY: CT	ZIP CODE: 06511
		SIGNATURE OF INVENTOR 204		DATE	
205	FULL NAME OF INVENTOR	LAST NAME: Fukumoto	FIRST NAME: Royd	MIDDLE NAME: K.	
	RESIDENCE & CITIZENSHIP	CITY: New York	STATE OR FOREIGN COUNTRY: New York	COUNTRY OF CITIZENSHIP: U.S.A.	
	POST OFFICE ADDRESS	STREET: 435 East 30th Street, #903	CITY: New York	STATE OR COUNTRY: NY	ZIP CODE: 10016
		SIGNATURE OF INVENTOR 205		DATE	

206	FULL NAME OF INVENTOR	LAST NAME: Zhang	FIRST NAME: Sheng	MIDDLE NAME:	
	RESIDENCE & CITIZENSHIP	CITY: New Haven	STATE OR FOREIGN COUNTRY: Connecticut	COUNTRY OF CITIZENSHIP: U.S.A.	
	POST OFFICE ADDRESS	STREET: 146 Cottage Street SE	CITY: New Haven	STATE OR COUNTRY: CT	ZIP CODE: 06511
		SIGNATURE OF INVENTOR 206		DATE	
207	FULL NAME OF INVENTOR	LAST NAME: Turenchalk	FIRST NAME: Gregory	MIDDLE NAME: S.	
	RESIDENCE & CITIZENSHIP	CITY: New Haven	STATE OR FOREIGN COUNTRY: Connecticut	COUNTRY OF CITIZENSHIP: U.S.A.	
	POST OFFICE ADDRESS	STREET: 820 Orange Street	CITY: New Haven	STATE OR COUNTRY: CT	ZIP CODE: 06511
		SIGNATURE OF INVENTOR 207		DATE	
208	FULL NAME OF INVENTOR	LAST NAME: Stewart	FIRST NAME: Rodney	MIDDLE NAME: A.	
	RESIDENCE & CITIZENSHIP	CITY: New Haven	STATE OR FOREIGN COUNTRY: Connecticut	COUNTRY OF CITIZENSHIP: U.S.A.	
	POST OFFICE ADDRESS	STREET: 512 Whitney Ave., Apt. B	CITY: New Haven	STATE OR COUNTRY: CT	ZIP CODE: 06511
		SIGNATURE OF INVENTOR 208			

DECLARATION FOR NON-PROVISIONAL PATENT APPLICATION*

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below at 201 et seq. beneath my name.

I believe I am the original, first and sole inventor if only one name is listed at 201 below, or an original, first and joint inventor if plural names are listed at 201 et seq. below, of the subject matter which is claimed and for which a patent is sought on the invention entitled

TREATMENT AND PREVENTION OF CANCER AND PITUITARY DISORDERS WITH LATS PROTEINS, DERIVATIVES AND FRAGMENTS, AND LATS KNOCK-OUT ANIMAL MODELS

and for which a patent application:
☐ is attached hereto and includes amendment(s) filed on *(if applicable)*
☒ was filed in the United States on February 16, 2001 as Application No. 09/763,334 *(for declaration not accompanying application)*
with amendment(s) filed on *(if applicable)*
☒ was filed as PCT international Application No. PCT/US99/19068 on August 18, 1999 and was amended under PCT Article 19 on *(if applicable)*

I hereby state that I have reviewed and understand the contents of the above identified application, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, §1.56.

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EARLIEST FOREIGN APPLICATION(S), IF ANY, FILED PRIOR TO THE FILING DATE OF THE APPLICATION			
APPLICATION NUMBER	COUNTRY	DATE OF FILING (day, month, year)	PRIORITY CLAIMED
			YES ☐ NO ☐
			YES ☐ NO ☐
			YES ☐ NO ☐

I hereby claim the benefit under Title 35, United States Code, §119(e) of any United States provisional application(s) listed below.

PROVISIONAL APPLICATION NUMBER	FILING DATE
60/096,996	August 18, 1998
60,096,997	August 18, 1998

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code §112, I acknowledge the duty to disclose information known to me which is material to patentability as defined in Title 37, Code of Federal Regulations, §1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application:

NON-PROVISIONAL APPLICATION SERIAL NO.	FILING DATE	STATUS		
		PATENTED	PENDING	ABANDONED

* for use only when the application is assigned to a company, partnership or other organization.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

201	FULL NAME OF INVENTOR	LAST NAME: Xu	FIRST NAME: Tian	MIDDLE NAME:	
	RESIDENCE & CITIZENSHIP	CITY: Guilford	STATE OR FOREIGN COUNTRY: Connecticut	COUNTRY OF CITIZENSHIP: U.S.A.	
	POST OFFICE ADDRESS	STREET: 149 Flag Marsh Road	CITY: Guilford	STATE OR COUNTRY: CT	ZIP CODE: 06437
		SIGNATURE OF INVENTOR 201		DATE	
202	FULL NAME OF INVENTOR	LAST NAME: Tao	FIRST NAME: Wufan	MIDDLE NAME:	
	RESIDENCE & CITIZENSHIP	CITY: Minneapolis	STATE OR FOREIGN COUNTRY: Minnesota	COUNTRY OF CITIZENSHIP: U.S.A.	
	POST OFFICE ADDRESS	STREET: Box 806 Mayo 420 Delaware Street SE	CITY: Minneapolis	STATE OR COUNTRY: MN	ZIP CODE: 55455
		SIGNATURE OF INVENTOR 202		DATE	
203	FULL NAME OF INVENTOR	LAST NAME: St. John	FIRST NAME: Maie	MIDDLE NAME: A.R.	
	RESIDENCE & CITIZENSHIP	CITY: Los Angeles	STATE OR FOREIGN COUNTRY: California	COUNTRY OF CITIZENSHIP: U.S.A.	
	POST OFFICE ADDRESS	STREET: 3008 Motor Avenue	CITY: Los Angeles	STATE OR COUNTRY: CA	ZIP CODE: 90064
		SIGNATURE OF INVENTOR 203		DATE	
204	FULL NAME OF INVENTOR	LAST NAME: Fei	FIRST NAME: Xiaolan	MIDDLE NAME:	
	RESIDENCE & CITIZENSHIP	CITY: New Haven	STATE OR FOREIGN COUNTRY: Connecticut	COUNTRY OF CITIZENSHIP: U.S.A.	
	POST OFFICE ADDRESS	STREET: 393 Mansfield Street, Apt. 21	CITY: New Haven	STATE OR COUNTRY: CT	ZIP CODE: 06511
		SIGNATURE OF INVENTOR 204		DATE	
205	FULL NAME OF INVENTOR	LAST NAME: Fukumoto	FIRST NAME: Royd	MIDDLE NAME: K	
	RESIDENCE & CITIZENSHIP	CITY: New York NY	STATE OR FOREIGN COUNTRY: New York	COUNTRY OF CITIZENSHIP: U.S.A.	
	POST OFFICE ADDRESS	STREET: 435 East 30th Street, #903	CITY: New York	STATE OR COUNTRY: NY	ZIP CODE: 10016
		SIGNATURE OF INVENTOR 205		DATE: April 23, 2001	

206	FULL NAME OF INVENTOR	LAST NAME: Zhang	FIRST NAME: Sheng	MIDDLE NAME:	
	RESIDENCE & CITIZENSHIP	CITY: New Haven	STATE OR FOREIGN COUNTRY: Connecticut	COUNTRY OF CITIZENSHIP: U.S.A.	
	POST OFFICE ADDRESS	STREET: 146 Cottage Street SE	CITY: New Haven	STATE OR COUNTRY: CT	ZIP CODE: 06511
		SIGNATURE OF INVENTOR 206		DATE	
207	FULL NAME OF INVENTOR	LAST NAME: Turenchalk	FIRST NAME: Gregory	MIDDLE NAME: S.	
	RESIDENCE & CITIZENSHIP	CITY: New Haven CT	STATE OR FOREIGN COUNTRY: Connecticut	COUNTRY OF CITIZENSHIP: U.S.A.	
	POST OFFICE ADDRESS	STREET: 820 Orange Street	CITY: New Haven	STATE OR COUNTRY: CT	ZIP CODE: 06511
		SIGNATURE OF INVENTOR 207: Gregory S. Turenchalk		DATE: 4/30/01	
208	FULL NAME OF INVENTOR	LAST NAME: Stewart	FIRST NAME: Rodney	MIDDLE NAME: A.	
	RESIDENCE & CITIZENSHIP	CITY: New Haven	STATE OR FOREIGN COUNTRY: Connecticut	COUNTRY OF CITIZENSHIP: U.S.A.	
	POST OFFICE ADDRESS	STREET: 512 Whitney Ave., Apt. B	CITY: New Haven	STATE OR COUNTRY: CT	ZIP CODE: 06511
		SIGNATURE OF INVENTOR 208			

DECLARATION FOR NON-PROVISIONAL PATENT APPLICATION*

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below at 201 et seq. beneath my name.

I believe I am the original, first and sole inventor if only one name is listed at 201 below, or an original, first and joint inventor if plural names are listed at 201 et seq. below, of the subject matter which is claimed and for which a patent is sought on the invention entitled

TREATMENT AND PREVENTION OF CANCER AND PITUITARY DISORDERS WITH LATS PROTEINS, DERIVATIVES AND FRAGMENTS, AND LATS KNOCK-OUT ANIMAL MODELS

and for which a patent application:
□ is attached hereto and includes amendment(s) filed on *(if applicable)*
☒ was filed in the United States on February 16, 2001 as Application No. 09/763,334 *(for declaration not accompanying application)*
with amendment(s) filed on *(if applicable)*
☒ was filed as PCT international Application No. PCT/US99/19068 on August 18, 1999 and was amended under PCT Article 19 on *(if applicable)*

I hereby state that I have reviewed and understand the contents of the above identified application, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, §1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, §119(a)-(d) of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

EARLIEST FOREIGN APPLICATION(S), IF ANY, FILED PRIOR TO THE FILING DATE OF THE APPLICATION			
APPLICATION NUMBER	COUNTRY	DATE OF FILING (day, month, year)	PRIORITY CLAIMED
			YES □ NO □
			YES □ NO □
			YES □ NO □

I hereby claim the benefit under Title 35, United States Code, §119(e) of any United States provisional application(s) listed below.

PROVISIONAL APPLICATION NUMBER	FILING DATE
60/096,996	August 18, 1998
60,096,997	August 18, 1998

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code §112, I acknowledge the duty to disclose information known to me which is material to patentability as defined in Title 37, Code of Federal Regulations, §1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application:

NON-PROVISIONAL APPLICATION SERIAL NO.	FILING DATE	STATUS		
		PATENTED	PENDING	ABANDONED

* for use only when the application is assigned to a company, partnership or other organization.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

201	FULL NAME OF INVENTOR	LAST NAME Xu	FIRST NAME Tian	MIDDLE NAME	
	RESIDENCE & CITIZENSHIP	CITY Guilford CT	STATE OR FOREIGN COUNTRY Connecticut	COUNTRY OF CITIZENSHIP U.S.A.	
	POST OFFICE ADDRESS	STREET 149 Flag Marsh Road	CITY Guilford	STATE OR COUNTRY CT	ZIP CODE 06437
		SIGNATURE OF INVENTOR 201		DATE May 7, 2001	
202	FULL NAME OF INVENTOR	LAST NAME Tao	FIRST NAME Wufan	MIDDLE NAME	
	RESIDENCE & CITIZENSHIP	CITY Minneapolis	STATE OR FOREIGN COUNTRY Minnesota	COUNTRY OF CITIZENSHIP U.S.A.	
	POST OFFICE ADDRESS	STREET Box 806 Mayo 420 Delaware Street SE	CITY Minneapolis	STATE OR COUNTRY MN	ZIP CODE 55455
		SIGNATURE OF INVENTOR 202		DATE	
203	FULL NAME OF INVENTOR	LAST NAME St. John	FIRST NAME Maie	MIDDLE NAME A.R.	
	RESIDENCE & CITIZENSHIP	CITY Los Angeles	STATE OR FOREIGN COUNTRY California	COUNTRY OF CITIZENSHIP U.S.A.	
	POST OFFICE ADDRESS	STREET 3008 Motor Avenue	CITY Los Angeles	STATE OR COUNTRY CA	ZIP CODE 90064
		SIGNATURE OF INVENTOR 203		DATE	
204	FULL NAME OF INVENTOR	LAST NAME Fei	FIRST NAME Xiaolan	MIDDLE NAME	
	RESIDENCE & CITIZENSHIP	CITY New Haven CT	STATE OR FOREIGN COUNTRY Connecticut	COUNTRY OF CITIZENSHIP U.S.A.	
	POST OFFICE ADDRESS	STREET 393 Mansfield Street, Apt. 21	CITY New Haven	STATE OR COUNTRY CT	ZIP CODE 06511
		SIGNATURE OF INVENTOR 204 Xiaolan Fei		DATE 05/09/2001	
205	FULL NAME OF INVENTOR	LAST NAME Fukumoto	FIRST NAME Royd	MIDDLE NAME K.	
	RESIDENCE & CITIZENSHIP	CITY New York	STATE OR FOREIGN COUNTRY New York	COUNTRY OF CITIZENSHIP U.S.A.	
	POST OFFICE ADDRESS	STREET 435 East 30th Street, #903	CITY New York	STATE OR COUNTRY NY	ZIP CODE 10016
		SIGNATURE OF INVENTOR 205		DATE	

206	FULL NAME OF INVENTOR	LAST NAME: Zhang	FIRST NAME: Sheng	MIDDLE NAME:	
	RESIDENCE & CITIZENSHIP	CITY: New Haven	STATE OR FOREIGN COUNTRY: Connecticut	COUNTRY OF CITIZENSHIP: U.S.A.	
	POST OFFICE ADDRESS	STREET: 146 Cottage Street SE	CITY: New Haven	STATE OR COUNTRY: CT	ZIP CODE: 06511
		SIGNATURE OF INVENTOR 206		DATE	
207	FULL NAME OF INVENTOR	LAST NAME: Turenchalk	FIRST NAME: Gregory	MIDDLE NAME: S.	
	RESIDENCE & CITIZENSHIP	CITY: New Haven	STATE OR FOREIGN COUNTRY: Connecticut	COUNTRY OF CITIZENSHIP: U.S.A.	
	POST OFFICE ADDRESS	STREET: 820 Orange Street	CITY: New Haven	STATE OR COUNTRY: CT	ZIP CODE: 06511
		SIGNATURE OF INVENTOR 207		DATE	
208	FULL NAME OF INVENTOR	LAST NAME: Stewart	FIRST NAME: Rodney	MIDDLE NAME: A.	
	RESIDENCE & CITIZENSHIP	CITY: New Haven	STATE OR FOREIGN COUNTRY: Connecticut	COUNTRY OF CITIZENSHIP: U.S.A.	
	POST OFFICE ADDRESS	STREET: 512 Whitney Ave., Apt. B	CITY: New Haven	STATE OR COUNTRY: CT	ZIP CODE: 06511
		SIGNATURE OF INVENTOR 208			

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APPLICATION NUMBER	COUNTRY	DATE OF FILING (day, month, year)	PRIORITY CLAIMED
			YES ☐ NO ☐
			YES ☐ NO ☐
			YES ☐ NO ☐

I hereby claim the benefit under Title 35, United States Code, §119(e) of any United States provisional application(s) listed below.

PROVISIONAL APPLICATION NUMBER	FILING DATE
60/096,996	August 18, 1998
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201	FULL NAME OF INVENTOR	LAST NAME: Xu	FIRST NAME: Tian	MIDDLE NAME:	
	RESIDENCE & CITIZENSHIP	CITY: Guilford	STATE OR FOREIGN COUNTRY: Connecticut	COUNTRY OF CITIZENSHIP: U.S.A.	
	POST OFFICE ADDRESS	STREET: 149 Flag Marsh Road	CITY: Guilford	STATE OR COUNTRY: CT	ZIP CODE: 06437
		SIGNATURE OF INVENTOR 201		DATE	
202	FULL NAME OF INVENTOR	LAST NAME: Tao	FIRST NAME: Wufan	MIDDLE NAME:	
	RESIDENCE & CITIZENSHIP	CITY: Minneapolis MN	STATE OR FOREIGN COUNTRY: Minnesota	COUNTRY OF CITIZENSHIP: U.S.A.	
	POST OFFICE ADDRESS	STREET: Box 806 Mayo 420 Delaware Street SE	CITY: Minneapolis	STATE OR COUNTRY: MN	ZIP CODE: 55455
		SIGNATURE OF INVENTOR 202: Wufan Tao		DATE: 5/3/07	
203	FULL NAME OF INVENTOR	LAST NAME: St. John	FIRST NAME: Maie	MIDDLE NAME: A.R.	
	RESIDENCE & CITIZENSHIP	CITY: Los Angeles	STATE OR FOREIGN COUNTRY: California	COUNTRY OF CITIZENSHIP: U.S.A.	
	POST OFFICE ADDRESS	STREET: 3008 Motor Avenue	CITY: Los Angeles	STATE OR COUNTRY: CA	ZIP CODE: 90064
		SIGNATURE OF INVENTOR 203		DATE	
204	FULL NAME OF INVENTOR	LAST NAME: Fei	FIRST NAME: Xiaolan	MIDDLE NAME:	
	RESIDENCE & CITIZENSHIP	CITY: New Haven	STATE OR FOREIGN COUNTRY: Connecticut	COUNTRY OF CITIZENSHIP: U.S.A.	
	POST OFFICE ADDRESS	STREET: 393 Mansfield Street, Apt. 21	CITY: New Haven	STATE OR COUNTRY: CT	ZIP CODE: 06511
		SIGNATURE OF INVENTOR 204		DATE	
205	FULL NAME OF INVENTOR	LAST NAME: Fukumoto	FIRST NAME: Royd	MIDDLE NAME: K.	
	RESIDENCE & CITIZENSHIP	CITY: New York	STATE OR FOREIGN COUNTRY: New York	COUNTRY OF CITIZENSHIP: U.S.A.	
	POST OFFICE ADDRESS	STREET: 435 East 30th Street, #903	CITY: New York	STATE OR COUNTRY: NY	ZIP CODE: 10016
		SIGNATURE OF INVENTOR 205		DATE	

206	FULL NAME OF INVENTOR	LAST NAME: Zhang	FIRST NAME: Sheng	MIDDLE NAME:	
	RESIDENCE & CITIZENSHIP	CITY: New Haven	STATE OR FOREIGN COUNTRY: Connecticut	COUNTRY OF CITIZENSHIP: U.S.A.	
	POST OFFICE ADDRESS	STREET: 146 Cottage Street SE	CITY: New Haven CT	STATE OR COUNTRY: CT	ZIP CODE: 06511
		SIGNATURE OF INVENTOR 206		DATE: 5/09/01	
207	FULL NAME OF INVENTOR	LAST NAME: Turenchalk	FIRST NAME: Gregory	MIDDLE NAME: S.	
	RESIDENCE & CITIZENSHIP	CITY: New Haven	STATE OR FOREIGN COUNTRY: Connecticut	COUNTRY OF CITIZENSHIP: U.S.A.	
	POST OFFICE ADDRESS	STREET: 820 Orange Street	CITY: New Haven	STATE OR COUNTRY: CT	ZIP CODE: 06511
		SIGNATURE OF INVENTOR 207		DATE:	
208	FULL NAME OF INVENTOR	LAST NAME: Stewart	FIRST NAME: Rodney	MIDDLE NAME: A.	
	RESIDENCE & CITIZENSHIP	CITY: New Haven	STATE OR FOREIGN COUNTRY: Connecticut	COUNTRY OF CITIZENSHIP: U.S.A.	
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		SIGNATURE OF INVENTOR 208		5/8/01	

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			YES □ NO □
			YES □ NO □

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	POST OFFICE ADDRESS	STREET: 149 Flag Marsh Road	CITY: Guilford	STATE OR COUNTRY: CT	ZIP CODE: 06437
		SIGNATURE OF INVENTOR 201		DATE	
202	FULL NAME OF INVENTOR	LAST NAME: Tao	FIRST NAME: Wufan	MIDDLE NAME:	
	RESIDENCE & CITIZENSHIP	CITY: Minneapolis	STATE OR FOREIGN COUNTRY: Minnesota	COUNTRY OF CITIZENSHIP: U.S.A.	
	POST OFFICE ADDRESS	STREET: Box 806 Mayo 420 Delaware Street SE	CITY: Minneapolis	STATE OR COUNTRY: MN	ZIP CODE: 55455
		SIGNATURE OF INVENTOR 202		DATE	
203	FULL NAME OF INVENTOR	LAST NAME: St. John	FIRST NAME: Maie	MIDDLE NAME: A.R.	
	RESIDENCE & CITIZENSHIP	CITY: Los Angeles CA	STATE OR FOREIGN COUNTRY: California	COUNTRY OF CITIZENSHIP: U.S.A.	
	POST OFFICE ADDRESS	STREET: 3008 Motor Avenue	CITY: Los Angeles	STATE OR COUNTRY: CA	ZIP CODE: 90064
		SIGNATURE OF INVENTOR 203: Maie AR St. John		DATE: 4/23/01	
204	FULL NAME OF INVENTOR	LAST NAME: Fei	FIRST NAME: Xiaolan	MIDDLE NAME:	
	RESIDENCE & CITIZENSHIP	CITY: New Haven	STATE OR FOREIGN COUNTRY: Connecticut	COUNTRY OF CITIZENSHIP: U.S.A.	
	POST OFFICE ADDRESS	STREET: 393 Mansfield Street, Apt. 21	CITY: New Haven	STATE OR COUNTRY: CT	ZIP CODE: 06511
		SIGNATURE OF INVENTOR 204		DATE	
205	FULL NAME OF INVENTOR	LAST NAME: Fukumoto	FIRST NAME: Royd	MIDDLE NAME: K.	
	RESIDENCE & CITIZENSHIP	CITY: New York	STATE OR FOREIGN COUNTRY: New York	COUNTRY OF CITIZENSHIP: U.S.A.	
	POST OFFICE ADDRESS	STREET: 435 East 30th Street, #903	CITY: New York	STATE OR COUNTRY: NY	ZIP CODE: 10016
		SIGNATURE OF INVENTOR 205		DATE	

206	FULL NAME OF INVENTOR	LAST NAME: Zhang	FIRST NAME: Sheng	MIDDLE NAME:	
	RESIDENCE & CITIZENSHIP	CITY: New Haven	STATE OR FOREIGN COUNTRY: Connecticut	COUNTRY OF CITIZENSHIP: U.S.A.	
	POST OFFICE ADDRESS	STREET: 146 Cottage Street SE	CITY: New Haven	STATE OR COUNTRY: CT	ZIP CODE: 06511
		SIGNATURE OF INVENTOR 206		DATE	
207	FULL NAME OF INVENTOR	LAST NAME: Turenchalk	FIRST NAME: Gregory	MIDDLE NAME: S.	
	RESIDENCE & CITIZENSHIP	CITY: New Haven	STATE OR FOREIGN COUNTRY: Connecticut	COUNTRY OF CITIZENSHIP: U.S.A.	
	POST OFFICE ADDRESS	STREET: 820 Orange Street	CITY: New Haven	STATE OR COUNTRY: CT	ZIP CODE: 06511
		SIGNATURE OF INVENTOR 207		DATE	
208	FULL NAME OF INVENTOR	LAST NAME: Stewart	FIRST NAME: Rodney	MIDDLE NAME: A.	
	RESIDENCE & CITIZENSHIP	CITY: New Haven	STATE OR FOREIGN COUNTRY: Connecticut	COUNTRY OF CITIZENSHIP: U.S.A.	
	POST OFFICE ADDRESS	STREET: 512 Whitney Ave., Apt. B	CITY: New Haven	STATE OR COUNTRY: CT	ZIP CODE: 06511
		SIGNATURE OF INVENTOR 208			

United States Patent & Trademark Office

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Application deficiencies found during scanning:

☐ Page(s)__________ of ______________________________ were not present for scanning. (Document title)

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☑ ***Scanned copy is best available.*** Figures 1A to 11 a are dark.